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MARINE BIOLOGICAL EFFECTS OF OCS PETROLEUM DEVELOPMENT

Douglas A. Wolfe, Editor

Outer Continental Shelf Environmental
Assessment Program
Boulder, Colorado
September 1978

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This document contains the proceedings of the first formal scientific review of the Biological Effects Studies managed by NOAA's Outer Continental Shelf Environmental Assessment Program (OCSEAP). The review was conducted November 29-December 1, 1977 at the Northwest and Alaska Fisheries Center in Seattle, Washington. The projects, managed by NOAA/OCSEAP, fall into two categories, based on the sources of financial support: Bureau of Land Management Environmental Studies Program in support of OCS leasing for Alaskan oil and gas development (Part I of Proceedings); NOAA's Energy Resources Project on Fate and Effects of Petroleum Hydrocarbons in Marine Ecosystems and Organisms, which is part of the Federal Interagency Energy/Environment Program, funded through the Environmental Protection Agency (Part II of Proceedings). Part III of the Proceedings contains discussions of selected topics addressed at the Review and a brief synopsis of general comments received from the invited outside reviewers. (Author extracted)

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MARINE BIOLOGICAL EFFECTS OF OCS PETROLEUM DEVELOPMENT

A Program Review of Research Supported Under the NOAA
Outer Continental Shelf Environmental Assessment Program
November 29 - December 1, 1977

Douglas A. Wolfe, Editor

Outer Continental Shelf Environmental
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September 1978

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MARINE BIOLOGICAL EFFECTS OF OCS PETROLEUM DEVELOPMENT

Interagency
Energy-Environment Research and Development
Program Report

U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
Environmental Research Laboratories

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PREFACE

This document contains the proceedings of the first formal scientific review of the Biological Effects Studies managed by NOAA's Outer Continental Shelf Environmental Assessment Program (OCSEAP). The review was conducted November 29-December 1, 1977 at the Northwest and Alaska Fisheries Center in Seattle, Washington.

The purposes of this program review were threefold:

1. To evaluate the past and recent results of the component research projects in terms of scientific quality and current national relevance, with special reference to assessment of potential environmental impacts of spilled petroleum in the marine environments. Such environmental assessment capability is required for decision-making for leasing Outer Continental Shelf (OCS) lands for oil and gas exploration and development, and for subsequent regulation during production phases of the OCS development.

2. To exchange recent results and progress to date among the various principal investigators involved in the program, as an improved basis for planning the next year's activities.

3. To discuss outstanding research needs and developing technology, which should be considered for future longer-term efforts.

Each investigator currently involved in the program presented a summary of objectives, recent progress, and future plans. Written versions of these presentations appear in this Proceedings. These projects, managed by NOAA/OCSEAP, fall into two categories, based on the sources of the financial support:

1. Bureau of Land Management Environmental Studies Program in support of OCS leasing for Alaskan oil and gas development (Part I of Proceedings);

2. NOAA's Energy Resources Project on Fate and Effects of Petroleum Hydrocarbons in Marine Ecosystems and Organisms, which is part of the Federal Interagency Energy/Environment Program, funded through the Environmental Protection Agency (Part II of Proceedings).

Part III of the Proceedings contains discussions of selected topics addressed at the Review and a brief synopsis of general comments received from the invited outside reviewers.

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PART I

STUDIES FUNDED UNDER THE BUREAU OF LAND MANAGEMENT OCS ENVIRONMENTAL STUDIES PROGRAM THROUGH THE NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION OUTER CONTINENTAL SHELF ENVIRONMENTAL ASSESSMENT PROGRAM

1. OCSEAP: AN OVERVIEW AND A CONTEXT FOR STUDIES ON BIOLOGICAL EFFECTS

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The Bureau of Land Management (BLM) initiated an extensive marine environmental studies program in response to national needs to assess the potential effects of OCS oil and gas development and to protect the environment. Through the offices of the Outer Continental Shelf Environmental Assessment Program (OCSEAP), the National Oceanic and Atmospheric Administration is planning and conducting for BLM that portion of the environmental studies program applicable to the OCS of Alaska. The origins, scope and current content of OCSEAP's efforts are described in earlier program plans (NOAA 1975), the current Program Development Plan (NOAA 1976) and a series of ten Technical Development Plans for fiscal year 1978 (NOAA 1977). I will attempt to provide here only a very brief overview of these extensive efforts in order to place the Biological Effects Program into proper perspective.

Nine Alaskan Outer Continental Shelf areas have been identified as having oil and gas potential, and consequently have appeared at one time or other on the official BLM leasing schedules (Figure 1). Environmental studies have been underway in each of these areas since 1974 (Northeast Gulf of Alaska), 1975 (Beaufort Sea, St. George Basin, Bristol Bay, Aleutians and Kodiak), or 1976 (Chukchi Sea, Norton Basin, and Lower Cook Inlet). In August 1977, BLM drastically revised the leasing schedule, and environmental studies in the postponed areas have been de-emphasized for FY 78 and subsequent years relative to those scheduled for early leasing.

For each lease area, depending on the priorities of the leasing schedule, the funds available, and the prior information on the area, OCSEAP has implemented a studies program designed to facilitate the assessment of potential OCS-related impacts. The studies program addresses six major tasks or questions, which together will enable BLM to proceed with OCS leasing, while minimizing the environmental risks of subsequent oil and gas development. Each of these major tasks has a series of component subtasks, which are addressed by a suite of field or laboratory studies contracted by OCSEAP with various Federal, state, university and private institutions. The six major tasks are listed below. For an extensive discussion of these tasks and component subtasks, see the Program Development Plan (NOAA 1976). Those interested in the specific composition of projects underway in each lease area may consult the Technical Development Plans (NOAA 1977) or the Program Work Statements (NOAA 1977). The Technical Development Plans are assembled

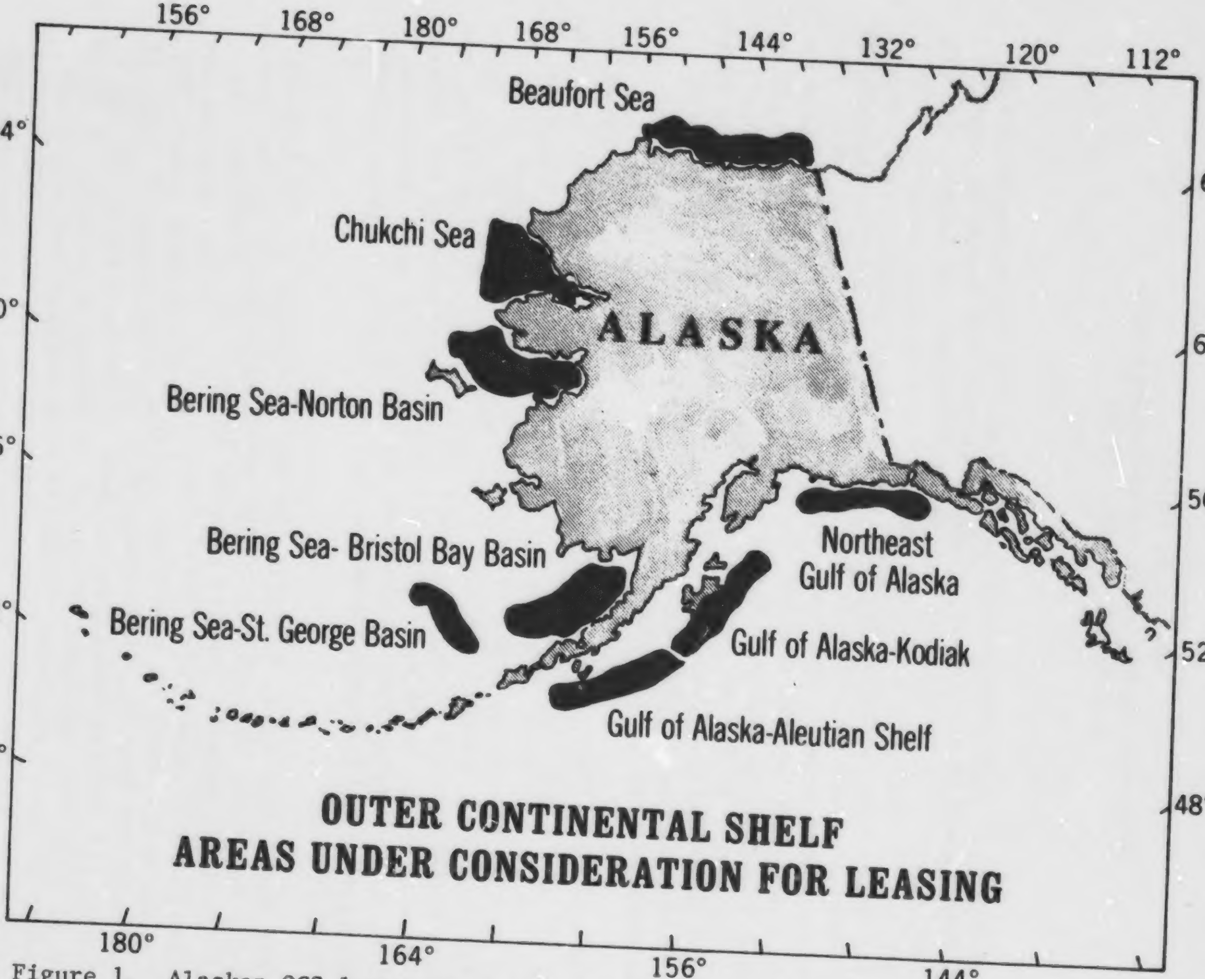

Figure 1. Alaskan OCS lease areas. As the August 1977 lease schedule from the Bureau of Land Management, sales are scheduled in only 5 areas: Beaufort Sea, December 1979; Gulf of Alaska, June 1980; Kodiak, October 1980; Cook Inlet, March 1981; and Norton Sound, December 1981. Additional sales in other areas are expected in subsequent years.

by program management as planning tools for solicitation of projects, whereas the Work Statements represent projects actually in place.

Table 1

Major Tasks Addressed by OCSEAP Studies

A. What is the existing distribution and concentration of potential contaminants associated with petroleum development?

B. What are the nature and magnitude of contaminants and environmental disturbances that may be assumed to accompany petroleum exploration and development?

C. What hazards does the environment pose to petroleum exploration and development?

D. How are contaminant discharges moved through the environment and altered by physical, chemical and biological processes?

E. What are the biological populations and ecological systems most subject to impact from petroleum exploration development?

F. What are the effects of contaminants and environmental alterations related to OCS oil and gas activities on individual organisms, populations, and ecological systems?

The relative funding emphasis received by the major tasks over the first four years of the program is shown in Table 2. For Task B, which focuses on obtaining the best available development scenarios in each lease area, OCSEAP relies on input from BLM and has expended no funds. The synthesis effort includes data management and is directed toward generation of reports and products for BLM use.

Table 2

OCSEAP Science Budget by Major Task or Discipline and Fiscal Year (Thousands of Dollars)

	Contaminant Baselines (A)	Hazards (C)	Transport (D)	Receptors (E)	Effects (F)	Synthesis	Total
FY 75	179	752	1,036	739	76	321	3,103
FY 76	1,500	2,569	2,831	7,162	1,009	592	15,663
FY 76T	145	366	281	979	99	141	2,011
FY 77	1,233	2,234	3,350	5,961	1,325	1,476	15,579
FY 78	994	1,683	3,554	5,145	1,306	1,359	14,051

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The phasing of studies implemented in response to OCSEAP's six major tasks is shown in Figure 2 for a "typical lease area." Obviously, the figure truely is not "typical" as only two lease sales have already occurred and both of these occurred only about 18 months after inception of the environmental studies. The scheme in Figure 2 does, however, represent the projected study sequencing for the lease areas over the next four years, if the current schedule is maintained.

In each geographic area, initial effort is placed on 1) general chemical characterization (pre-development contaminant concentrations; 2) assessment of hazards to development; 3) transport studies (mainly physical oceanographic trajectory analysis); and 4) location and characterization of biological resources. This information is available in time for draft and final environmental impact statements (Figure 2). As the sale occurs and exploratory drilling and development proceed, attention will be shifted increasingly to site-specific studies, based on the results of best available scenarios for development, including probable locations of rigs, pipelines and shoreside facilities. These site-specific studies will probably be more and more inshore as the development scenarios unfolded, biological resources are located, and probable trajectories become known.

Concurrent with field studies directed towards Tasks A-E is a primarily laboratory effort on Task F, the biological effects task. Coupled together, the first five tasks allow OCSEAP and BLM to assess the probability of impingement of development upon ecological and biological resources. The effects studies on the other hand should enable us to evaluate the probable consequences of that impingement. To date the effects studies have consisted mainly of laboratory experiments, i.e., acute and chronic challenge experiments wherein organisms are somehow exposed to contaminants and the resultant effects are measured. OCSEAP has visualized, however, controlled experiments under field conditions as well, to test effects on ecological systems not readily maintained in the laboratory and to verify laboratory observations under field conditions.

Effects studies tend to be non-area-specific in terms of applicability. Results may be applied to any lease are in which the test organisms or the target process occurs. The component subtasks which make up Task F are listed in Table 3 and discussed further in the Non-Site-Specific Technical Development Plan (NOAA 1977). These eight subtasks represent the general information needs which have been identified during continue program planning, and they provide the context in which the effects projects to date were solicited and selected. Three additional subtasks, F-9 to F-11, described in the TDP, represent integrated site-specific assessments and are not under the purview of this review.

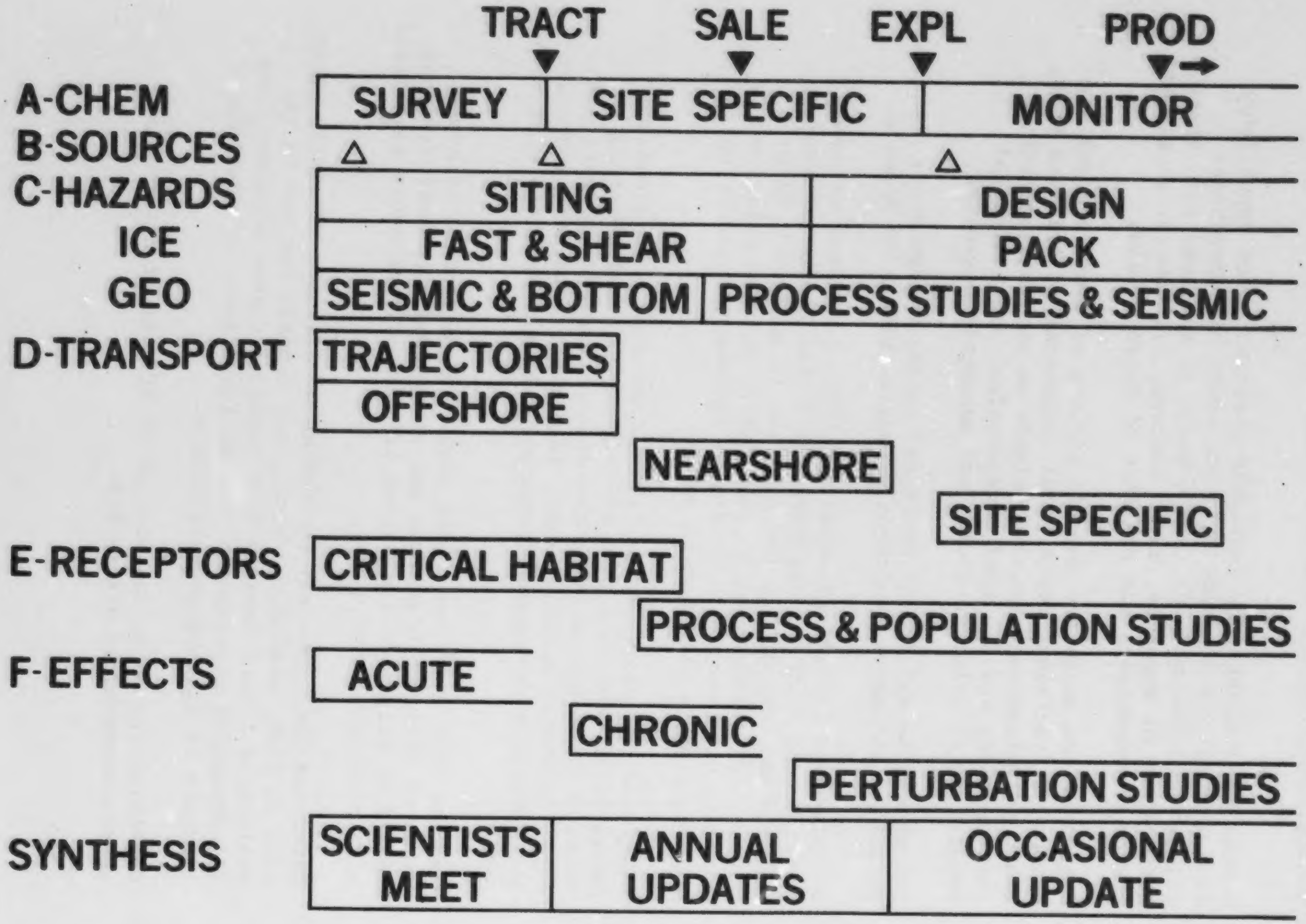

Figure 2. Phasing of OCSEAP study elements in relation to the steps in development of a lease area: tract selection, lease sale, exploratory drilling and production.

Table 3

Subtasks Addressed in OCSEAP's Biological Effects Studies

F-1 Review and evaluate the available literature and unpublished data on toxicity of crude oils and crude oil components (including toxic metals) on the basis of species, life stage, temperature at exposure, water sources, oil source, geographic source of organisms, and presence of toxic metals.

F-2 Determine the acute and chronic effects of crude oil and its component fractions, toxic metal components of drilling muds, other petroleum-associated chemicals on survival, growth, reproduction, and selected physiological and behavioral mechanisms of selected arctic and subarctic organisms.

F-3 Determine the effects of crude oil on thermoregulatory mechanism and other functions of marine birds and mammals.

F-4 Determine by laboratory experiments the potential release of toxic metals from oil-impacted sediments, the occurrence of soluble and non-soluble toxic metals in sediments (with emphasis on organometallic complexes), and the relative importance of these toxic metals on various species in terms of uptake and effects on biota.

F-5 Determine by laboratory experiment the bioaccumulation and relative effects of petroleum hydrocarbons and other OCS-related contaminants presented through various exposure pathways, including soluble forms, food chain exposure, suspended-particulate-borne contaminants and sediment-absorbed contaminants.

F-6 Conduct laboratory and field studies to determine recovery rates of selected organisms and ecosystems from perturbations caused by either contamination or other disturbances associated with petroleum development.

F-7 Determine the types and incidences of diseases presently occurring in fish, shellfish, birds, and mammals for use in (A) evaluating future impacts of petroleum-related activity, and (B) designing experiments to test the effects of contaminant exposure to disease-susceptibility.

F-8 Determine the potential ecological effects of alternative countermeasures to oil spills.

Table 4 summarizes the research projects which have been implemented in response to the preceding eight subtasks. All of those research units (RU's) receiving support in FY 78 were presented at the effects review and are described within this proceedings. Two other research units, RU's 29/30 and RU 190 address microbiological environmental characterization objectives primarily, but in addition are examining effects of microbes on petroleum composition or effects of petroleum exposure on heterotrophic microbial function. These research units, headed by R. Atlas and R. Morita, respectively, are also included in this review proceedings, even though they are discussed in area-specific TDP's apart from the remaining effects studies.

Reports of the effects studies completed in FY 77 or before are included in the annual or quarterly compilations of Principal Investigators' Reports published by OCSEAP (NOAA 1976). A primary achievement has been the publication of the final report of RU 75 which is a thorough review of available literature through 1976 on the effects of oil on artic and subarctic organisms (Malins 1977).

In summary then, this review is intended to provide a basis for evaluating how well OCSEAP's Biological Effects Program so far has responded to the programmatic subtasks, and for recommending research areas for increased or decreased emphasis in future years.

Table 4

Summary of Effects Research Projects - OCSEAP

RU #	Principal Investigator	Title	Subtasks Addressed	Funding ($K) FY 75	FY 76	FY 77	FY 78
62	Arthur L. Devries Phys. Research Lab. Scripps Inst. of Ocean.	The Physiological Effect of Acute and Chronic Exposure to Hydrocarbons and of Petroleum on the Nearshore Fishes of the Bering Sea	F-2	9.0	38.7	--	--
71	G. L. Kooyman Scripps Institute	Physiological Impact of Oil on Pinnipeds	F-3	--	91.5	59.0	60.0
72	S. D. Rice J. F. Karinen NMFS/Auke Bay Fisheries	Acute and Chronic Toxicity, Uptake, and Depuration and Sublethal Metabolic Response to Alaskan Marine Organisms to Petroleum Hydrocarbons	F-2 F-8	27.8	275.6	500.0	300.0
73	D. C. Malins NMFS/NWAFC	Sublethal Effects as Reflected by Morphological, Chemical, Physiological and Behavioral Indices	F-2 F-6 F-7	--	153.9	combined 360.0	400.0
74	D. C. Malins NMFS/NWAFC	Identification of Major Processes in Biotransformations of Petroleum Hydrocarbons and Trace Metals	F-2 F-6	--	76.5		
75	D. C. Malins NMFS/NWAFC	Assessment of Available Literature on Effects of Oil Pollution on Biota in Arctic and Subarctic Waters	F-1	--	33.0	--	--
96	S. Patten Johns Hopkins Univ.	Efforts of Petroleum Exposure on Hatching Success and Chick Survival of Alaskan Glaucous-Winged Gulls	F-3	( )	( )	(28.9)	(30.0)

Table 4 (continued)

RU #	Principal Investigator	Title	Subtasks Addressed	Funding ($K)			
				FY 75	FY 76	FY 77	FY 78
123	Ronald L. Smith IMS/Univ. of Alaska	Acute Effects - Pacific Herring Roe in Gulf of Alaska	F-2	—	62.0	—	—
183	Richard S. Caldwell Oregon State Univ. of Marine Science	Acute and Chronic Toxicity of Seawater Extracts of Alaskan Crude Oil to Zoeae of the Dungeness Crab, *Cancer magister*, Dana	F-2	31.3	7.4	—	—
278	Robert Barsdate IMS/Univ. of Alaska	Microbial Release of Soluble Trace Metals from Oil-Impacted Sediments	F-4	6.2	17.2	—	—
305	John G. Pearson/ Peter McRoy IMS/Univ. of Alaska	Sublethal Effects - Effects on Seagrass Photosynthesis	F-2	—	77.7	—	—
332	B. B. McCain NMFS/NWAFC	Incidence of Pathology in the Gulf of Alaska, Bering Sea, and Beaufort Sea	F-7	—	111.1	53.6	47.3
389	Jeannette Whipple NMFS Tiburon Laboratory	Transport, Retention and Effects of Toxic Petroleum Hydrocarbons in Experimented Food Chains	F-5	—	86.1	163.9	15.0
404	J. W. Anderson Battelle Marine Lab.	Accumulation of Organis and Heavy Metals from Petroleum-Impacted Sediments by Marine Detritivores	F-2 F-4 F-6	—	41.5	108.0	117.0
423	C. R. Grau Pt. Reyes Bird Observatory	Influence of Petroleum on Egg Formation and Embryonic Development in Seabirds	F-3	—	—	12.8	30
500	J. Scott Warner Battelle Columbus	Activity-Directed Fractionation of Petroleum	F-2	—	—	75.5	100.0

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National Oceanic and Atmopsheric Administration 1977. Technical Development Plan - FY 78. Environmental Assessment of the Alaskan Continental Shelf. In 10 volumes: Beaufort Sea; Shukchi Sea; Bering Sea; Norton Basin; Bering Sea - Bristol Bay; Bering Sea - St. George Basin; Gulf of Alaska - Aleutian Shelf; Gulf of Alaska - Kodiak; Lower Cook Inlet; Northeast Gulf of Alaska; Non-Site Specific.

National Oceanic and Atmospheric Administration 1977. Program Work Statements FY 1977. Environmental Assessment of the Alaskan Continental Shelf. July 1977. 867 pp.

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2. ACUTE AND CHRONIC TOXICITY, UPTAKE AND DEPURATION, AND SUBLETHAL RESPONSE OF ALASKA MARINE ORGANISMS TO PETROLEUM HYDROCARBONS

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Oil effects studies at the Auke Bay Laboratory began in late 1971 with minimal funding. In 1974, Shell Oil, later joined by five other oil companies, supplied the first significant quantities of operational funds for an 18 month study on oil effects, in response to the lack of toxicity information on marine organisms of commercial interest from Kachemak Bay, or on any Alaskan species in general. At about this time, Fish and Wildlife Serfice began funding a smaller project that measured oil toxicities and effects on fresh water fishes, in response to the need for information associated with the Trans-Alaska Pipeline. These sources of funding produced six completed papers (one review, one on methods, and four on effects) and the beginning of several, later completed or added to by OCSEAP funding. OCSEAP funding began in the last 2 months of FY 75, continued at significant funding levels through FY 76, 77, and will continue through at least FY 78. This report describes progress associated with OCSEAP funding only, and draws primarily from published or drafted manuscripts, although unwritten but completed studies up to October 1977 are mentioned.

Our studies can be broken down into two basic themes: (1) Toxicity challenge experiments, where we attempt to identify sensitive species, life stages, factors that affect toxicity, or components that are most responsible for toxicity, and (2) sublethal physiological response, where we attempt to identify, measure, and characterize physiological responses that are indicative of oil stress. Eventually these sublethal studies will provide information useful in identifying exposed animals in the field, an evaluation of how stressed the exposed animals are, and possibly the mode or mechanism of toxic action. We often conduct uptake-depuration studies in parallel to the above tests, to correlate tissue concentrations with effects, which will aid in the interpretation of results.

TOXICITY CHALLENGE EXPERIMENTS

COMPARATIVE SENSITIVITY OF ALASKAN MARINE ORGANISMS

We have tested over 30 species of Alaska marine organisms to crude oil and No 2 fuel oil. TLm values for most of these species (4 fish, 6 crustaceans and other invertebrates) are reported in Rice, Short, Karinen (1976) with an updated-final version due in 1978. The challenge tests identified two basic groups of marine organisms: sensitive and tolerant. The sensitive group were mostly subtidal species which included all the fish and crustaceans. The tolerant species were mostly intertidal invertebrates such as snails, mussels, etc. There were some exceptions in both groups, i.e. mysids were tolerant and limpets and chitons were sensitive. The sensitivity of the intertidal limpets and chitons may be due in part to the collecting procedure, since they were pried off the substrate when collected. The effect of collecting was demonstrated in a field experiment where survival of marked limpets, collected and returned to the same collection site (no exposure), was less than controls that were marked but not collected. The tolerance of the intertidal species is probably related to their normal physiological adaptation to shutdown and the ability to isolate themselves from the environment during a normal low tide. These test were all static, with concentrations declining with time, and many of the animals could probably isolate themselves from the exposure media until concentrations had declined considerably.

COMPARISON OF STATIC AND FLOW-THROUGH TESTS

To determine the validity and reliability of static tests for comparing animal sensitivites to oil, we exposed two sensitive species (pink salmon fry and Eualis shrimp) and two tolerant species (black cucumber and Hemigrapsus shore crab) to static and flow-through exposures of toluene and naphthalene. The two sensitive species had flow-through 96 h TLm's that were about 70% of the TLm's determined with static exposures. The tolerant species flow-through 96 h TLm's that were about 35% of the TLm's determined with static exposures. These data suggest that comparative sensitivites of diverse species require flow-through exposures, that tolerant species are not that tolerant under flow-through conditions, and that static tests are not that bad for some species if you consider the cost/benefit ratio. (Manuscript in preparation).

ARE ALASKAN SPECIES MORE SENSITIVE THAN ANIMALS FROM WARMER CLIMATES

Sensitivities were compared in a review (Rice, Short, Karinen 1977). Although Alaskan species had low 96 h TLm's than tests reported in the literature for animals from warmer waters, differences in species, temperature, and oils tested precluded direct-conclusive comparisons. Since the data compared was all static, we hypothesized that the differences observed were not species differences (as in more sensitive), but differences in toxicity caused by temperature. At warmer temperatures, static tests would lose aromatic hydrocarbons at faster rates (thus higher initial concentrations are required to be equally toxic), in contrast to colder temperatures where toxicity persists longer. This stimulated later studies on temperature effects on toxicity-sensitivity.

THE EFFECT OF TEMPERATURE ON TOXICITY-SENSITIVITY

Loss of Aromatics from WSF's at Different Temperatures

As a companion experiment to toxicity at different temperatures (see below), we measured the losses of individual aromatic hydrocarbons (by GC) at different temperatures (4°, 8°, and 12° C) and under different conditions (with and without aeration, with and without microbial poisons). In general, increased temperatures caused increases in rates of loss. The primary cause of loss for most of the volatile monoaromatics was evaporation, as shown by aeration. In contrast, larger percentages of the di-aromatics were lost through biodegradation. (Manuscript in preparation, delays have been caused by the death of the senior author).

Comparative Sensitivity at Different Temperatures

We are conducting this experiment in two ways: (1) static, with declining concentrations with time and at different rates for each temperature, and (2) flow-through, with stable concentrations. Each experiment is with pink salmon fry and shrimp tested at 4°, 8°, and 12°C.

Static tests (Korn, Moles, and Rice--submitted to Journal) found lower TLm's at lower temperatures for the pink salmon fry, to be expected since lower temperatures cause toxic hydrocarbons to persist longer. However, lower TLm's at higher temperatures were found for shrimp, indicating they were more sensitive at higher temperatures. Temperature can affect toxicity-sensitivity measurements in three ways: (1) by affecting the persistence of the toxic aromatic hydrocarbons, (2) by affecting the sensitivity of the animal to oil stress by altering internal physiological processes, and (3) extreme temperatures will be an additional stress, independently or possibly synergistically.

Some flow-through tests have been completed, and do not appear to be significantly different from the static tests. Uptake tests at different temperatures have also been completed, but have not been analyzed.

SENSITIVITY OF COMPARATIVE LIFE STAGES: FISH

Herring Eggs and Larvae

Larvae were relatively sensitive while eggs were tolerant to WSF's of crude oil. (unpublished)

Salmonid Smolts: Fresh Water vs Seawater

We have tested several species and life stages in fresh water, under contract to Fish and Wildlife Service, and found emergent fry to be more sensitive than sac fry and eggs. When this series of life stage tests was continued with smolts, with some tested in fresh water while others were tested in seawater (OCSEAP funded) a dramatic shift in sensitivity occurred. Seawater acclimated fish (normal habitat) were about twice as sensitive to WSF and pure compounds. The test with pure compounds (benzene) ruled out the possibility that the effect was caused by a subtle shift in hydrocarbon composition of the WSF between tests in fresh water vs seawater. (Manuscript in lab review--Moles, Korn, and Rice)

SENSITIVITY OF COMPARATIVE LIFE STAGE: CRUSTACEA

Comparative Sensitivity of Stage I Shrimp and Crabs

Larvae were found to die more slowly than adults, and when moribund (no swimming, random pleopod twitching) they would exist for days, appearing equal in sensitivity to adults if TLm's were determined on the basis of no motion death. However, sensitivities of larvae (based on observations of moribund larvae) were approximately an order of magnitude greater than the adults. The range of 96 h TLm for moribundity for six species of stage I shrimp and crab was relatively small--0.95 to 0.7 ppm. (Broderson et al. 1977)

Comparative Sensitivity of Stage I-VI Coonstripe Shrimp

Sensitivity varied considerably between stages (III and VI most sensitive; I and IV most tolerant), indicating that species comparisons based on stage I may not concern the most sensitive stage, and that comparing between stages of different species may not be valid. There was a larger range between stages of one species (.24-1.9 ppm) than there was between species at stage I (.95-1.9 ppm). (Broderson et al. 1977)

Comparative Sensitivity of Intermolt and Molting Larvae

Molting larvae of shrimp and crab were more sensitive to WSF than intermolt larvae (before and after molt). This indicates that comparison of sensitivities between species must consider the stage of molt or large differences may be observed in error. (Mecklenburg et al. 1977)

Uptake of Hydrocarbons into Intermolt and Molting Larvae

Bioassays with pure compounds of toluene and naphthalene were required to identify lethal and sublethal exposure levels. These tests confirm the earlier observations with crude oil WSF that molting larvae were more sensitive than intermolt larvae. Uptake of toluene and naphthalene was rapid, reaching equilibrium with each toxicant in less than an hour. Simple adsorption to the outer skin was ruled out when dead larvae (killed by O_2 privation for 1 h) did not take up the isotope at fast rates, and when molting larvae (previously exposed and placed in clean water prior to molting) carried the labeled hydrocarbons with the tissue and did not leave significant quantities in the discarded exoskeleton. Toluene was not depurated by the larvae as quickly as naphthalene. (Manuscript in prep.)

Long-Term Exposure to Shrimp Eggs. Egg carrying females were exposed 30 days to static exposures of WSF that were replenished daily. If the adult females lived, the eggs hatched and viable larvae resulted. (Manuscript in preparation)

IDENTIFICATION OF THE MAJOR TOXIC COMPOUNDS OR FRACTION IN WSF OF OIL

The water soluble fraction of crude oil is composed of many aromatic and aliphatic hydrocarbons. Previous literature has indicated that the aromatics cause most of the toxicity of a WSF; however, which aromatics contribute how much to the total toxicity is unknown. We approached the problem with three experimental designs with the ultimate goal of assessing the relative toxicity contribution of the aromatics in the water soluble fraction of crude oil. We hope eventually to be able to predict the toxicity of a WSF based on the concentration of important aromatics in the WSF and the research results from this series of studies.

Role of Phenols, Naphthols, and Heterocycles in Toxicity of WSF's

We determined the toxicity role of phenols, naphthols, and heterocycles to shrimp and pink salmon by determining: (1) quantities in oil and WSF's, (2) the acute toxicity of the compounds found in the highest concentration, and (3) the availability to the organism by conducting uptake-depuration experiments for the major compounds. Water and oil samples were analyzed by Don Brown at NOAA National Analytical Facility in Seattle: naphthols were not found and low levels of phenols and cresols were found (30-80 ppb) in the WSF of oil and fuel oil. Bioassays demonstrated phenol and cresol to be mildly toxic to shrimp and fish (96 h TLm 3-12 ppm). Uptake studies showed phenol and cresol accumulated 10-20 times the water concentration in 24 h, then the compounds were depurated by 4-13 days. The low concentration of phenols in WSF plus the relatively low toxicity and accumulation indicate that phenols are not important contributors to the acute toxicity of an oil WSF. (Manuscript in preparation)

Toxicity of WSF's and Synthetic WSF's

When analyzing WSF by GC, approximately 6-10 aromatic hydrocarbons are detected in concentrations that are presumed to be most responsible for the toxicity observed. We are attempting to conduct flow-through tests of WSF;s and synthetic WSF to see if the two toxicities are equivalent. The synthetic WSF is composed of the same major aromatic hydrocarbons, and in the same relative concentrations. If the toxicities are equal, then many compounds that are not detected in significant concentrations can be presumed to be relatively unimportant. These tests are now in progress.

Synergism Studies

We have started assaying important oil aromatics in pairs with shrimp and fish using continuous-flow apparatus. The object is to determine whether the toxicity of the two compounds is additive, synergistic, or antagonistic. This information is needed to allow prediction of the toxicity of a WSF based on GC chemical analyses. Sublethal experiments strongly suggest that the mode of toxicity for mono- and dinuclear aromatic hydrocarbons are not the same, indicating that the toxicities may not be additive.

SUBLETHAL EFFECTS STUDIES

RESPONSE OF MACOMA CLAMS TO WSF AND OIL CONTAMINATED SEDIMENT

A study by Taylor and Karinen (1977) determined the organism response and short-term toxicity of crude oil layered on mud, dissolved in water, and adsorbed in sediment to Macoma balthica. Although few deaths were noted during 72 h exposures, a surfacing response of clams was noted when exposed to either dissolved of sediment adsorbed oil. Responses were directly proportional to concentrations of dissolved oil. Responses were directly proportional to concentrations of dissolved oil or amount of oil treated sediment. These results suggest that behavioral responses of clams to oil may be of great importance to their survival since clams that come to the sediment surface may be eaten by predators or die from exposure. Results helped to explain disappearance of clams in field-oil exposure experiments by Shaw et al. (1976).

THE EFFECT OF AROMATIC HYDROCARBONS ON BYSSAL THREAD FORMATIONS IN THE MUSSEL MYTILUS EDULIS

Mussels extrude small but measurable quantities of byssal threads continuously to insure their attachment to rocky substrates that are pounded by surf. We exposed mussels to toluene, naphthalene, and WSF of crude oil to determine if byssal thread extrusion would be impaired at sublethal concentrations. Exposed mussels had a significantly lower rate of byssal thread formation when compared to control mussels. The significance of this finding is that the ability of mussels to maintain populations in exposed rocky areas may be reduced when exposed to oil and the turbulent forces from waves. Settling of young mussels in these areas would likely be inhibited. (Manuscript in preparation, Taylor, Rice, and Karinen)

EFFECT OF AROMATIC HYDROCARBONS ON OXYGEN CONSUMPTION AND BREATHING RATES OF PINK SALMON FRY

Rice and Thomas have completed a series of three studies to determine the effects of hydrocarbon exposure on breathing rates in pink salmon fry.

In the first paper of this series, Thomas and Rice (1975) showed that opercular rates increased significantly for as long as 9 h and 12 h after exposure to the water soluble fractions of Prudhoe Bay crude oil. The increase in rates was proportional to the increase in dose and the observed changes occurred at approximately 20% of the 96 h TLm. Recording changes in opercular rates appears to be a suitable method for detecting and quantitating sublethal physiological effects of stress.

Rice, Thomas, and Short (1977) in a follow-up paper determined that dissolved hydrocarbons from two crude oils and No. 2 fuel oil caused similar increases in breathing and coughing rates in pink salmon fry. Response was proportional to dose for each oil and naphthalene was believed to be the common component responsible for the response. Response again occurred at about 20-30% of the 96 h TLm and remained above normal during a constant dose for 72 h. Breathing rates were highest during the first 24 h when hydrocarbons were being metabolized and/or excreted. A continued elevated breathing rate at somewhat reduced level from the high indicates that salmon fry can cope with a sublethal exposure to hydrocarbons, but at the cost of an increased metabolic rate. Tissue concentrations of naphthalene and subtituted naphthalene correlated well with metabolic response.

Thomas and Rice (submitted to Journal) proved the relationship between oxygen consumption (mtabolic rate) and opercular breathing rates of pink salmon fry exposed to toluene, naphthalene and water soluble fractions of Cook Inlet crude oil and No. 2 fuel oil. Data from this study indicated that opercular breathing rate reflects oxygen consumption and therefore is directly related to metabolic rate. Breathing rate was again proportional to dose and showed a linear response to toluene and naphthalene, although the threshold of response was much greater for toluene than naphthalene. Temperature had a pronounced effect on the response to some toxicants but not to others. Increases in breathing rates were about the same (38-48%) with all toxicants at 12° C. However, at 4° C naphthalene and No. 2 fuel oil caused a great increase (more than 100%) while toluene and Cook Inlet crude oil gave increases of 50-55%. Toluene appeared to be more toxic at 4° C (when the increased response was low), but the toxicity of other toxicants was unaffected by temperature.

Results from this series of sublethal effects studies may have application to monitoring effects of oil and defining the mode of action of toxicants. Monitoring breathing rates of fry from areas suspected of being chronically polluted with hydrocarbons could provide evidence along with chemical baseline data that there will be an effect on growth and possibly survival. These data are providing clues as to the mode of action of various toxicants and form the basis for further experimentation.

THE PATHWAY OF ELIMINATION FROM FISH OF AROMATIC HYDROCARBON

Fish were exposed to labeled toluene or naphthalene via ingested capsules, and placed in "split chambers" which separated the "gill" area from the "anal" area. Samples were analyzed to determine quantity and form (parent hydrocarbon or metabolite). Toluene was excreted primarily from the gill, with peak excretion rates prior to 8 h, and the isotope was primarily in the form of the "Parent Toluene" rather than as metabolite. Naphthalene was not excreted significantly within 24 h. Most of the naphthalene was found in the tissues, with the highest concentrations found in the liver and gall bladder, which also had the highest percentage of metabolite. (Manuscript in preparation, Thomas and Rice)

EFFECT OF AROMATIC HYDROCARBON EXPOSURES ON CRAB HEAT RATES AND OXYGEN CONSUMPTION

Heart rates in king crab were measured after exposure to Cook Inlet crude oil, benzene, and naphthalene for the purpose of finding a physiological parameter, easily measured, which was indicative of metabolic response. Heart rates, measured by electrocardiogram, were inversely proportional to aromatic hydrocarbon exposure (opposite of salmon fry response). Heart rates declined rapidly during the first hours of exposure, then returned to control or near control rates as toxicant concentrations in test-waters declined. If the toxicant concentration was maintained stable at a high level, however, the depression of heart rates persisted. Changes in oxygen consumption closely paralleled the changes in heart rate, confirming that heart rate is a sensitive index of metabolic reponse to toxicants in king crab. (Manuscript in preparation. Mecklenburg and Rice)

Heart rate determinations in crabs from areas of suspected oil pollution by spill or chronic accumulation may have application in proving the biological impact of given hydrocarbon concentrations and evaluating the extent of such impact. These data will also have application in determining the mode of action of toxicants in crustaceans, and determining the co-effects of temperature and toxicant on metabolic rates.

EFFECT OF AROMATIC HYDROCARBONS ON CELL MORPHOLOGY OF EXPOSED KING CRAB AND PINK SALMON FRY

Changes in king crab gill tissue morphology were identified by transmission electron microscopy after short-term (3 day) exposure to the water soluble fraction of Cook Inlet crude oil. Cell damage was extensive with (1) extensive vacuolization, (2) extremely electron dense nucleii, (3) electron dense cytoplasm, (4) fewer mitochondria with blebbing, (5) smaller rough endoplasmic reticulum cisternae, and (6) distorted interdigitations along lateral and basal cell surfaces. Cell damage was not apparent in samples collected 3 h after the beginning of exposure but was apparent at 6 days following retention of animals for 3 days in clean water. (Borchek and Babcock, in Lab Review)

Changes in cell surface morphology of olfactory epithelium from pink salmon fry were identified by scanning electron microscopy after exposure to sublethal concentrations of benzene. Following 12 days exposure to benzene concentrations as low as 0.15 ppm mucous cell activity increased and cilia distribution was altered on the olfactory lamellar surfaces. These alterations in olfactory tissue may interfere with chemosensory reception and consequently interfere with homing, migratory routes, and feeding activity. (Babcock, in Lab Review)

Changes in cell morphology following oil exposure may be particularly useful in monitoring the impact of oil in the environment.

RECOMMENDATIONS FOR FUTURE RESEARCH

TOXICITY

1a. There is a need to determine the effects of temperature and salinity on the toxicity of hydrocarbons.

b. The relationship between toxicity of various crude oil components and temperature appears to be complex with either synergistic or antagonistic effects resulting, depending upon component and species tested.

c. Information on the effects of temperature and salinity on the toxicity of crude oil components will aid in predicting the impacts of spills under various environmental conditons. (Arctic spills, open oceans, estuaries, etc.).

2a. The synergistic effect of hydrocarbons needs further study.

b. Various crude oil components apparently have different action sites, some additive some not, and different entry and exit routes and rates.

c. Information on interaction of component toxicity will aid in predicting the toxicity of various crude oils.

3a. Dispersant oil tests with selected representative Arctic spills need to be conducted.

b. Information on the toxicity of the new type dispersants is lacking, especially with Arctic species. Tests should be made with species previously challenged to the oil alone.

c. Information will assist in evaluating the impact of dispersant use in Arctic and Subarctic Ocean spills.

4a. Several selected species representative of previously tested temperate and subarctic species should be bioassayed.

b. Temperature appears to have a significant effect on the toxicity of various hydrocarbons and the effect also appears to be species-dependent.

c. Information will assist in determining whether toxicity data collected on Alaskan species so far can be extended to Arctic species.

5a. A number of potentially sensitive species and life stages of Alaskan marine organisms are yet to be tested. Groups particularly needing toxicity bioassays are: fish eggs and larvae, noncrustacean invertebrate eggs and larvae, pelagic larvae which settle on mud/sand sediments, and fish species which burrow in sand or sediment.

b. Larvae are the most sensitive of the life stages tested. Insufficient data are available to determine the sensitivities of larvae of various groups.

c. Information will aid in the determinations of the impact of spills during periods of meroplankton abundance.

6a. Long-term 30-day exposures should be tested on about six species.

b. Tests should be run on both sensitive and resistant species as determined by acute static bioassays to ascertain their relative sensitivity on a long-term basis.

c. Information obtained may allow some generalizations about the relative sensitivities of various groups and the application of bioassay data to estimate real environmental impacts.

SUBLETHAL EFFECTS

1a. The effects which temperature and salinity have on observed physiological perturbations resulting from oil exposure should be tested.

b. The sensitivities of certain species to hydrocarbons is very dependent on temperature and salinity. Is this sensitivity reflected in sublethal physiological changes? Correlation of tissue hydrocarbon loads with tissue damage is needed.

c. Information will assist in evaluating impact of Arctic spills and chronic addition of hydrocarbons to Arctic environments, both oceanic and estuarine.

2a. Recovery rates of organisms from sublethal perturbations should be determined.

b. Little is known about the duration of time that the normal physiological acitivites of an organisms exposed to a single dose of hydrocarbons are inhibited or changed.

c. Information on recovery rates will assist in evaluating and monitoring the impact from one-time oil exposure.

3a. Effects studies which gave positive or negative results should be repeated with selected Arctic species.

b. This will help to define the roles of temperature and species differences in determining hydrocarbon sensitivity.

c. Information will determine whether results from the data based on temperate and subarctic organisms can be applied to Arctic species.

4a. Quantitate uptake and sublethal effects for organisms exposed to oil contaminated sediments.

b. Hydrocarbon concentrations in sediments are very high compared to water and organisms in close association with sediments are likely to develop physiological perturbations as a result of this close contact.

c. Information will assist in evaluating the impact of given concentrations of hydrocarbons observed in the environment.

ADAPTATION OF ARCTIC SPECIES TO OIL

1a. Select an Arctic species which has a short generation time. Rear parallel colonies in the laboratory, one exposed to oil and one not exposed to oil. After several generations challenge both control and exposed organisms to oil determining toxicity, sublethal effects, metabolism of oil, etc. Determine the effects of aged oil on both groups.

b. Evidence of successful reproduction of selected organisms around natural oil seeps in temperate regions (Santa Barbara) suggest that marine organisms may adapt to oil. How fast do they adapt? Do Arctic species adapt as readily?

c. Information will be of significant value in evaluating the impact of chronic oil additions in Arctic regions.

CORRELATION OF LABORATORY STUDIES WITH FIELD EXPOSURES

1a. Utilizing spills of opportunity or areas subject to chronic addition of oil correlate tissue burdens, effects and toxicity observed in laboratory exposures with tissue burdens, effects and toxicity observed in field exposures to hydrocarbons.

b. A comparison of the effects of real environmental exposures with the effects of exposures under laboratory conditions is needed before the results of laboratory studies can be applied to the field situation.

c. Information will define utility of laboratory studies.

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3. SUBLETHAL EFFECTS OF PETROLEUM HYDROCARBONS AND TRACE METALS INCLUDING BIOTRANSFORMATIONS, AS REFLECTED BY MORPHOLOGICAL PHYSIOLOGICAL, PATHOLOGICAL, AND BEHAVIORAL INDICES

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INTRODUCTION

The findings described in the following papers on the biological fate of petroleum (including trace metals) and related alterations in organism viability were obtained through a multidisciplinary research effort of the Environmental Conservation Division. The organisms included representative species of pelagic and demersal fish and invertebrate organisms of arctic and subarctic waters. Such animals, exposed to petroleum under various conditions reflecting the field situation, were examined for biological alterations through the application of procedures associated with disciplines, such as pathology, cell biology, physiology, and biochemistry. The broad disciplinary approach to the assessment of altered viability provided a favorable opportunity for discovering critical alterations in life systems.

In the early phases of our work, because of limited data available on the nature of alterations wrought by petroleum, we were obliged to spend considerable effort in simply identifying petroleum-related changes. This work increased our understanding of the nature of biological changes and where they tended to occur in the organisms. We are now focusing much of our effort on identifying biological alterations on a concentration/exposure time-dependent basis.

Over a period of about two years, we have been able to relate various types of petroleum exposures to alterations in the life processes of key subarctic and arctic organisms. Moreover, through our biochemical studies, we have provided substantial evidence to show that petroleum ingested by most marine organisms is rapidly and extensively transformed to metabolic products of potentially high toxicity. These findings and others are presented in five overview papers. The authors have paid special attention to interpreting our findings in terms of the needs of environmental policy makers and regulatory agencies, and have further described their views about areas of research which should be pursued in the near future.

DISPOSITION AND METABOLISM OF PETROLEUM HYDROCARBONS

Donald C. Malins

OBJECTIVES AND RATIONALE

The objectives of this aspect of the program are to evaluate the disposition and metabolism of petroleum hydrocarbons in marine organisms indigenous to Alaskan waters. Experimental designs include the exposure of organisms to water-soluble fractions (WSF) of petroleum, to petroleum in the diet, and to individual aromatic hydrocarbons containing radioactive labels. Exposed animals are studied with respect to the uptake, accumulation, and metabolism of persistent low levels of hydrocarbons arising from experimentally contaminated environments.

The question of the formation and retention of metabolites in the tissues of petroleum-exposed marine organisms is of critical importance because of a substantial body of evidence indicating that electrophilic products of aromatic hydrocarbon metabolism bring about deleterious alterations in animal tissues (Malins, 1977; Varanasi and Malins, 1977). Overall, data acquired on the study of the metabolism of petroleum aromatic hydrocarbons in arctic and subarctic marine organisms is being related, through other phases of our program, to alterations in organism viability. Thus, the data on metabolism open up the possibility of relating alterations in the health of organisms to the type, concentration, and duration of exposure to petroleum in the environment. Such associations are presently being made and will continue as the research proceeds.

Studies conducted on this program so far have addressed the questions given above. The findings obtained have significantly enhanced our understanding of the dispositon, metabolism, and biological effects of petroleum in pelagic and demersal marine fish, as well as in larval and postlarval invertebrates.

METHODS

ORGANISMS EMPLOYED

The fish used were coho salmon (Oncorhynchus kisutch) and starry flounder (Platichthys stellatus); invertebrates included spot shrimp (Panadalus platyceros) and coon-stripe shrimp (P. hypsinotus).

EXPOSURE CONDITIONS

Coho salmon and starry flounder were exposed to 0.9 ± 0.1 ppm of a water-soluble fraction (WSF) of Prudhoe Bay crude oil in flowing seawater. The flow-through system, designed by Roubal et al. (1977a), was rich in the more volatile components of the crude oil, such as toluene, m-xylene, and a variety of methyl-substituted aromatic hydrocarbons containing one and two benzenoid rings. The virtual absence of water-insoluble alkanes in the flow-through water implied that organisms were exposed only to water-soluble aromatic hydrocarbons, rather than to a system containing occluded oil particles.

Coho salmon were also exposed to individual isotopically-labeled hydrocarbons, such as benzene, naphthalene, and anthracene in the diet, in flowing water, and through intraperitoneal injection. The latter technique was generally employed to obtain sufficient incorporated radioactivity to permit the detection and identification of small concentrations of metabolic products.

Larval and postlarval invertebrates were exposed to ^{14}C- and ^{3}H-labeled naphthalene under flow-through seawater conditions to study the metabolism of this hydrocarbon.

CHEMICAL ANALYSES

Concentrations of total hydrocarbons and individual components of the WSF were assayed via gas liquid chromatography of solvent extracts; structural determinations were made via mass spectrometry. Isotopically-labeled aromatic hydrocarbons were obtained in high chemical- and radio-purity from commercial suppliers. The purity of these compounds was checked in our own laboratories. Quantitative evaluations of isotopically-labeled hydrocarbons in the diet, in seawater, and in tissues were undertaken via liquid scintillation spectrometry of solvent extracts.

Animals exposed to isotopically-labeled hydrocarbons were studied with respect to both the accumulation of the parent hydrocarbons and the formation and retention of metabolic products. Hydrocarbons and total metabolic products extracted from tissues were separated via solvent partition techniques described by Roubal et al. (1977b) and Varanasi et al. (1977). These methods allowed for the solubilization of the hydrocarbon in hexane and the assay of total metabolic products by determining the total radioactivity in either an acidic or basic aqueous phase. Individual metabolites were isolated from tissue extracts employing thin-layer chromatographic techniques (Roubal et al. 1977b). Also, improved resolution of individual metabolites is now being obtained through the use of high-pressure liquid chromatography using a methanol-water elution system (Collier et al. 1977; Thomas et al. 1978).

RESULTS

EXPOSURES THROUGH WATER COLUMN

The accumulation of low molecular weight aromatic hydrocarbons by coho salmon and starry flounder was examined using a WSF of Prudhoe Bay crude oil (Roubal et al. 1978). The animals were exposed to 0.9 ± 0.1 ppm of the WSF. Coho salmon and starry flounder were subjected to these conditions for six and two weeks periods, respectively. After the exposure, both species were transferred to clean seawater for two weeks to study the depuration of accumulated hydrocarbons.

Hydrocarbons in the WSF comprised low molecular weight volatile aromatic compounds, i.e., benzene, toluene, xylenes, other alkylated benzenes and naphthalene, and alkylated naphthalenes.

Seven analyses of the WSF were performed during the course of the exposure.

No hydrocarbons from the WSF were detected in coho salmon tissues after one week of exposure. After two to six weeks, however, significant amounts were found (Fig. 1). After five weeks (the time of maximum hydrocarbon accumulation), bioconcentration factors (ppm hydrocarbons based on dry weight of tissue/ppm hydrocarbon in flow-through water) for C_3-substitued benzenes, naphthalene, the combined 1- and 2-methylnaphthalenes, C_2-substituted naphthalenes, and C_3-substituted naphthalenes were 50, 80, 160, 85, and 140, respectively. The C_4/C_5-substituted benzenes, present at about 0.01 ppm in the WSF, comprised the major portion of the hydrocarbons in the coho salmon. Muscle contained 5.5 ppm of the C_4/C_5-substituted benzene fraction after five weeks, equivalent to a bioconcentration factor of 550. After six weeks a significant decline in all hydrocarbon accumulations was obtained in coho salmon muscle, possibly because of the induction of the hydrocarbon-metabolizing enzymes and alterations in rates of excretory processes. When exposed coho salmon were placed in clean water, no evidence was found in muscle tissue for hydrocarbons of the WSF after a period of one week.

In contrast to coho salmon, where no petroleum hydrocarbons were detected after one week of exposure, relatively high levels of the hydrocarbons (e.g., 93 ppm of the combined C_4-/C_5-substituted benzenes) were identified in starry flounder muscle in this time period (Fig. 1). Hydrocarbon bioconcentrations in muscle were as follows: C_3-substituted benzenes, 500; combined C_4-/C_5-substituted benzene fracion, 9,300; naphthalene, 700; combined 1- and 2-methylnaphthalenes, 2,400; C_2-substituted naphthalenes, 2,400; and C_3-substituted naphthalenes, 3,400.

Substantial bioconcentrations of hydrocarbons occurred in liver and gills throughout the exposures. The C_4-/C_5-substituted benzenes in starry flounder were 1-2 orders of magnitude greater than in coho salmon. After two weeks these hydrocarbons attained the highest concentration in liver

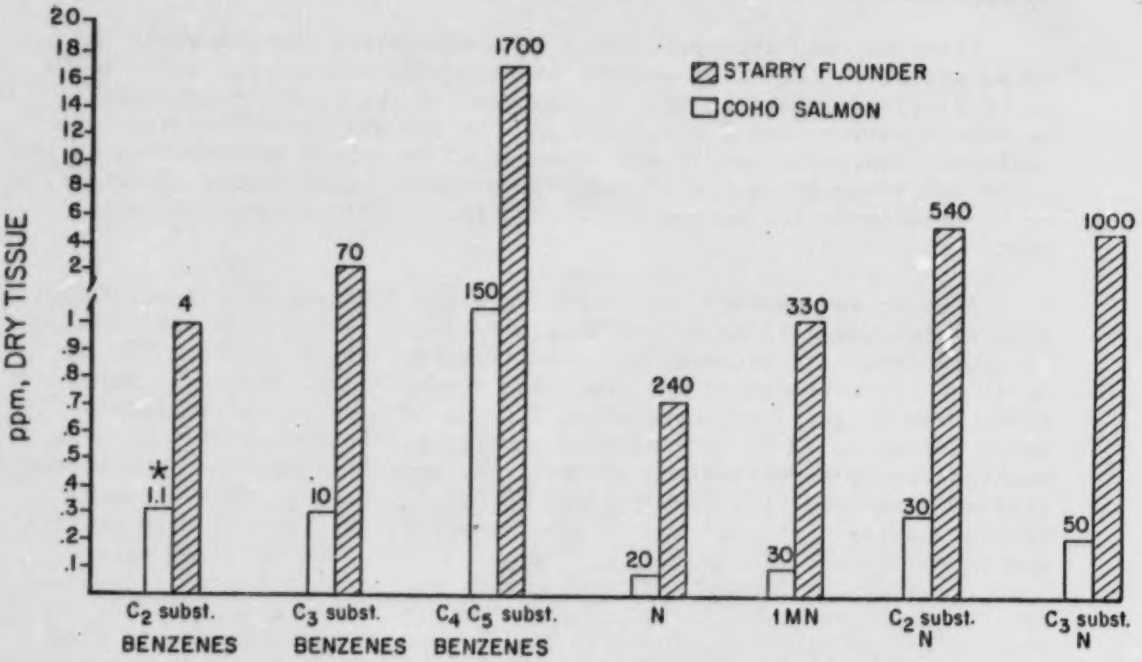

Figure 1. Hydrocarbons in muscle tissue of coho salmon and starry flounder exposed two weeks to a WSF of crude oil (from Roubal et al., 1978).

*Bioaccumulation factors.

(110 ppm), representing a bioconcentration factor of 11,000. Concentrations of hydrocarbons up to C_3-substituted naphthalenes increased in each tissue in relation to the degree of alkylation. Similar conclusions can be drawn from the data on exposed coho salmon. A significant decline in all individual hydrocarbons occurred in the muscle of exposed starry flounder after two weeks. Over the same period the liver of starry flounder showed an increase in hydrocarbon accumulations. No comparable changes were detected in the gills.

After exposed starry flounder were maintained for two weeks in clean seawater, levels of several of the hydrocarbons were still found to be elevated in muscle tissue: 26 ppm for the C_4-/C_5-substituted benzene fraction, and 7.0 and 8.0 ppm for C_2- and C_3-substituted naphthalenes, respectively. Concentrations of petroleum hydrocarbons in the gills and liver of starry flounder were either below limits of detection or at relatively low levels two weeks after termination of the experiment.

A study was carried out to evaluate the tendency of salmonids to accumulate hydrocarbons and metabolic products in the skin when exposed to hydrocarbons in surrounding water (Varanasi et al. 1977). The results of the water-immersion study are summarized as follows: Skin, taken from S. gairdneri exposed to 9.2 ppb of naphthalene in flowing water for up to 72 hr, accumulated substantial concentrations of both naphthalenes and metabolic products. The concentrations of naphthalene reached a maximum (4,230 ppb) after 48 hr of exposure, then began to decline during the remaining 24 hr of exposure (2,100 ppb). It continued to decline for 72 hr (223 ppb) during depuration in clean water. Concentrations of the metabolite in skin continued to increase throughout the exposure, reaching a value of 441 ppb at the end of 72 hr. At the end of the exposure, 15.8% of the total radioactivity in the skin was attributable to a metabolite fraction. After 72 hr of depuration in clean water, metabolites still constituted 52% of the total radioactivity in the skin.

Skin of S. gairdneri from the water-immersion study accumulated substantially larger amounts of both naphthalene and its metabolites than skin from either the force-feeding or injection studies. Livers from the water-immersion study also contained considerably higher radioactivity than livers from force-feeding and injection studies conducted under this program.

In an attempt to understand the extent to which marine larval invertebrates accumulate and metabolize water-borne hydrocarbons, we exposed spot shrimp (Pandalus platyceros) and Dungeness crab (Cancer magister) larvae to 8-12 ppb concentrations of ^{14}C-labeled naphthalene and ^{14}C-labeled naphthalene complexed with bovine serum albumin (BSA) in separate tests (Sanborn and Malins, 1977). The hydrocarbon was complexed with protein to determine whether aromatic components of petroleum are metabolizing differently when associated with macromolecules.

The ^{14}C-labeled naphthalene and naphthalene-BSA complex produced 100% mortality in 24-36 hr in newly hatched Dungeness crab and Stage I and V spot shrimp. Maximum accumulation of radiolabeled naphthalene in Stage V spot shrimp was nearly four times greater than in shrimp exposed to the naphthalene-BSA complex (820 and 220 ppb, respectively). These values represent a bioconcentration of 25-100 times the exposure levels. Metabolic products of naphthalene in larval tissues exposed to the complexed and noncomplexed naphthalene reached a maximum of 21 and 9%, respectively, of the total radioactive compounds (based on the molecular weight of 1-naphthol). It is particularly noteworthy that the naphthalene was almost entirely depurated from the tissues in 12 hr regardless of the chemical form, whereas total metabolic products showed no appreciable change during depurations up to 24 hr. The nature of the individual metabolites in invertebrate larvae exposed to high specific activity ^{3}H-naphthalene is presently being determined. These results confirm our earlier findings that significant amounts of metabolic products are formed in invertebrate larvae exposed to naphthalene and that the individual metabolic products can be identified via chromatographic techniques.

Challenge experiments on postlarval shrimp were conducted using ^{3}H-naphthalene with a high specific activity, which facilitates identification of very small amounts of individual metabolites (Sanborn, unpublished). Postlarval shrimp (P. hypsinotus) were exposed for 144 hr in continuous flowing seawater containing 6 ppb of ^{3}H-naphthalene. Depuration was evaluated after 48 hr in clean water. The postlarval shrimp, reared from ovigerous females captured in the vicinity of Kodiak Island, were found to accumulate 360 ppb of naphthalene and 20 ppb of total metabolites (expressed as 1-naphthol) in 1 hr. The naphthalene concentration in the shrimp reached 650 ppb in 3 hr. During the exposure period, the naphthalene concentration declined from a high of 740 ppb at 12 hr to 46 ppb at 72 hr, possibly due to the induction of metabolizing enzymes or enhanced excretion. Subsequently, the concentrations of naphthalene increased somewhat in the remaining 72 hr of exposure. Metabolites reached a maximum concentration of 50 ppb in 24 hr. Metabolite concentrations, however, decreased only slightly during the remainder of the exposure. During depuration studies, the naphthalene concentration decreased from 198 ppb after 144 hr of exposure to 35 ppb in 6 hr; but metabolite concentrations only changed from 50 to 35 ppb in this period. No mortalities were recorded during the exposure and depuration phases of the experiment.

In another experiment (Sanborn, 1977), one-year old spot shrimp were exposed to the WSF of Prudhoe Bay crude oil at a concentration of 0.9 ppm in seawater for seven days. The WSF was obtained from the solubilizer designed by Roubal et al. (1977a). The findings indicated that detectable levels of low molecular weight aromatic hydrocarbons are readily accumulated in thoracic segments of the animals. In contrast, abdominal segments were found to contain no more than trace levels of aromatic hydrocarbons (Table 1). The highest accumulations of aromatic hydrocarbons were found with C_2-substituted benzenes (25 ng/g wet wt); however, methyl-substituted naphthalenes were also present in the thoracic segments (15-19 ng/g wet wt).

Table 1. Hydrocarbons in thoracic and abdominal segments of one-year-old spot shrimp exposed to water-soluble fraction of Prudhoe Bay crude oil using flow-through system[a]

Compound	Thoracic segments (ng/g wet wt)	Abdominal segments (ng/g wet wt)
C_2-Substituted benzenes	25	Trace[b]
C_3-Substituted benzenes	17	Trace
C_4- & C_5-Substituted benzenes	2	Trace
Naphthalene	1	Trace
1- & 2-Methylnaphthalenes	19	Trace
C_2-Substituted naphthalenes	18	Trace
C_3-Substituted naphthalenes	15	Trace

[a] 0.11 ppm total hydrocarbons in flow-through water.

[b] Trace - below measurable limit. (From Sanborn, 1977)

Exposure Through the Diet

To study the disposition of aromatic hydrocarbons in pelagic species exposed to these compounds in the diet, we administered 5 μCi of ^{14}C-labeled naphthalene and anthracene via the diet to coho salmon in separate tests (Roubal et al. 1977b). The findings indicated, with one exception (muscle, from naphthalene exposure, 72 hr), that substantially more ^{14}C from anthracene remained in the tissue in comparison to naphthalene. In this particular experiment, the ^{14}C was associated with both the hydrocarbon and aromatic products of metabolism. After 24 hr, the brain, muscle, and liver of the anthracene-fed fish comprised 363, 572, and 546% more ^{14}C, respectively, than comparable tissues from the naphthalene-fed fish. Maximum levels of radioactivity appeared in the liver and brain of the anthracene-fed fish 24 hr after termination of feeding. These concentrations represent 0.02% of the administered ^{14}C in brain, and 0.05% in liver. Taking into account the depletion of ^{14}C from 24 to 168 hr (the sharpest declines in the values obtained) the loss of radioactivity associated with naphthalene and metabolic products was 98 and 95% for liver and muscle, respectively, and 98% for brain. Anthracene-fed animals showed a comparable depletion from brain, liver, and muscle of 79, 79, and 86%, respectively.

An additional experiment on the disposition of naphthalene in pelagic fish was carried out with coho salmon receiving both ^{14}C- and

^{3}H-labeled naphthalene in separate tests through the diet (Collier et al. 1977). In this case, the aromatic hydrocarbon was administered in herring oil because data in the literature imply that aromatic hydrocarbons in marine organisms are primarily sequestered by lipid deposits. Because of the extremely high solubility of aromatic hydrocarbons in lipids, we anticipated that this experiment strongly favored the direct excretion of the aromatic hydrocarbon as opposed to absorption through the alimentary canal. There were two important features to this experiment: an attempt was made to evaluate the disposition of naphthalene in coho salmon at a high (10°C) and a low (4°C) temperature regime to evaluate differences in retention relating to arctic versus temperate waters. Further, an attempt was made to determine whether differences in metabolite formation could be found in relation to temperature using the solvent partition techniques. In this work we applied high pressure liquid chromatography for the first time to the isolation and identification of individual metabolites formed in exposed marine organisms (Collier et al. 1977).

We found that decreased temperature (4° vs 10°) resulted in a significantly ($P<0.05$) increased retention of the dietary ^{14}C-labeled naphthalene. This increased retention occurred in brain, liver, kidney, and blood after 16 hr of exposure. Our attempts to investigate whether environmental temperature influenced the formation and retention of naphthalene metabolites resulted only in the identification of such compounds in the gall bladder, but no significant differences in relation to temperature were obtained. The sensitivity of the partition method was about 160 ng of total metabolites/g of dry tissue. Metabolic products in the gall bladder were detected only in the 16 hr exposure regime. At 4° and 10°C, the average values for three fish ±s.e.m. were found to be 510 ± 330 ng/g and 480 ± 330 ng/g, respectively. Our inability to detect total metabolites in other organs was attributed to the mode of administration (triglyceride oil), restricting passage across the intestine, and to the relatively low specific activity of the commercially available ^{14}C-labeled naphthalene.

To determine whether metabolites could be detected at concentrations below the limit of detectability of the solvent partition method, we repeated the experiment using specially synthesized high specific activity ^{3}H-naphthalene. Solvent extracts of pooled tissues from special fish were subjected to high pressure liquid chromatography. The results confirmed the presence of at least 10 individual metabolites of naphthalene (five have been structurally identified thus far) in the liver and gall bladder 16 hr after feeding the ^{3}H-naphthalene (Fig. 2). The principal derivative was the dihydrodiol, which was found in liver and gall bladder to the extent of 110 picomoles/g and 200 picomoles/g, respectively.

Studies are presently underway on the metabolism of 2,6-dimethylnaphthalene in coho salmon (Roubal, Lazaran, and Malins, unpublished). This hydrocarbon, administered through the diet, has been found to

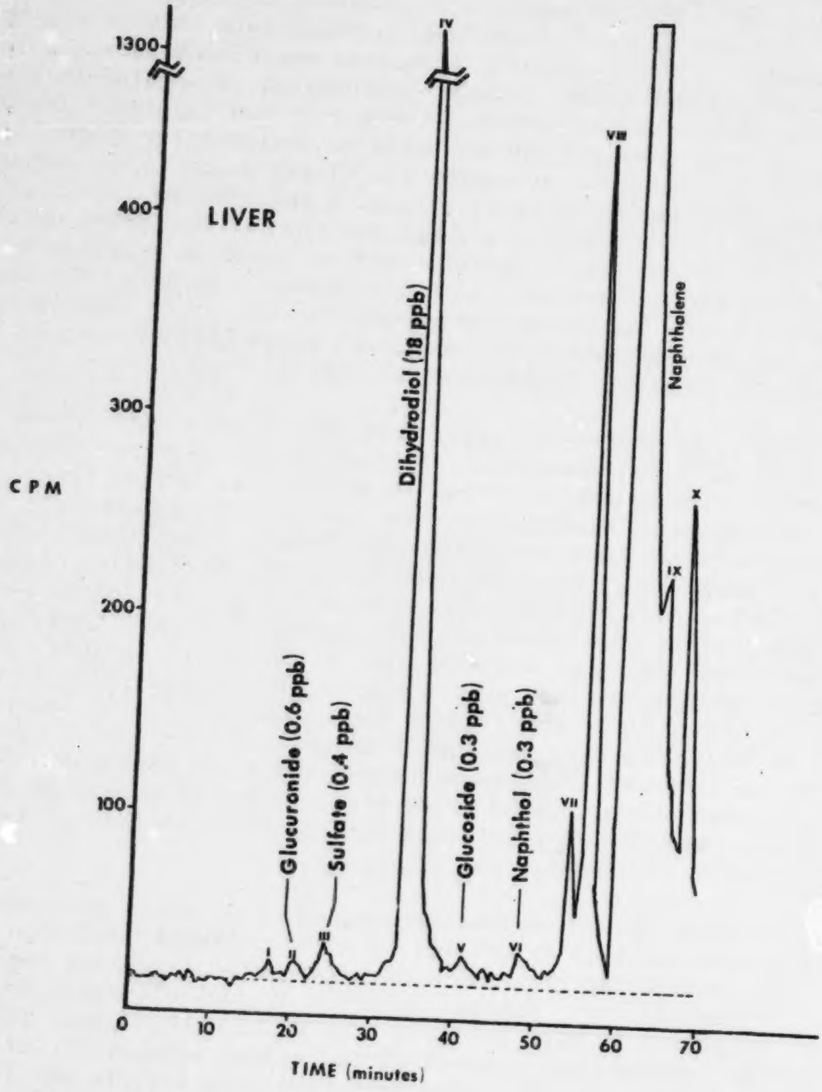

Figure 2. HPLC profiles of metabolites of naphthalene in liver of coho salmon after force-feeding of 112 μg ^{3}H-naphthalene (from Collier et al., 1978).

accumulate substantially in tissues of pelagic fish and our present findings, though preliminary, indicate that coho salmon are able to convert this alkylated hydrocarbon to a variety of metabolic products. At present, the products have not been positively identified. The study with the 2,6-dimethylnaphthalene will soon be extended to starry flounder because of our previous work showing that these organisms have a remarkably high capacity to bioconcentrate alkyl-naphthalenes. Recent studies (Varanasi and Gmur, 1977) show that starry flounder fed ^{3}H-naphthalene, accumulate considerable amounts of naphthalene in liver, skin, and blood. More importantly, substantial amounts of metabolites were present in all tissues investigated, 24 hr after feeding naphthalene. For example, at 24 hr, liver, blood skin, and bile contained 29, 62, 45, and 87% respectively, of the radioactive metabolities and at 168 hr, the values increased to 81, 87, 75, and 98%. Thus, it is apparent that demersal fish, such as starry flounder, are able to extensively metabolize aromatic hydrocarbons and store them in tissues.

The skin of marine fish is a site of a number of known lesions including tumors (McCain, 1977). Because the skin of marine fish appears to be particularly susceptible to cellular damage, possibly associated with environmental pollution (Hodgins et al. 1977), a study was carried out on the disposition of naphthalene and its metabolites in the skin of S. gairdneri force-fed this hydrocarbon in the diet (Varanasi et al. 1977). Concentrations of both naphthalene and its metabolites in the skin increased steeply with time, reaching maximum values of 322 and 17 ppb, respectively, at 24 hr and subsequently declined to 10 and 2.5 ppb, respectively, at 168 hr. Statistical treatment of the data showed that rates of change in concentration of naphthalene and its metabolites were significantly different ($P<0.05$): Naphthalene concentrations increased more rapidly than the metabolites. Thus, at 24 hr 95.6% of the total radioactivity in skin was represented by naphthalene. There was, however, a rapid decrease in naphthalene concentration at 168 hr; the metabolites represented 17.7% of the total radioactivity of the skin at this time, as shown by the solvent partition technique. At each time of sampling, epidermal mucus of the test fish contained considerably larger concentrations of metabolites than naphthalene. Concentrations of naphthalene and metabolites were 45 and 130 ppb, respectively, at 24 hr after force-feeding.

In studies of the disposition of 1-methylnaphthalene (Varanasi and Gmur, 1977) in the skin of saltwater-adapted coho salmon, substantial amounts of 1-methylnaphthalene (931 ppb) and its metabolites (86 ppb) were found 24 hr after force-feeding. At 24 hr the skin of the test fish contained 4.1% of the administered dose of 1-methylnaphthalene, of which 7.1% was attributable to metabolites. At 48 hr, however, as much as 38.5% of the total radioactivity in the skin was in the form of metabolites.

As with S. gairdneri, the epidermal mucus of the saltwater coho salmon exposed to 1-methylnaphthalene contained relatively high concentrations of metabolites with respect to the parent hydrocarbon up to 48 hr after the initial retament. Epidermal mucus of starry flounder (Varanasi and Gmur, 1977) fed naphthalene contained considerably larger concentration of the metabolites compared to naphthalene. It appears from these results (Varanasi et al. 1977; Varanasi and Gmur, 1977) that skin and epidermal mucus play an important role in accumulation, discharge, and retention of petroleum hydrocarbons and their metabolites. This fact should be taken into account when considering overall metabolism and excretion of xenobiotics in aquatic organisms.

EXPOSURE THROUGH INTRAPERITIONEAL INJECTION

In an attempt to maximize the incorporation of hydrocarbon into body tissues, we studied the distribution of ^{14}C-labeled benzene, naphthlene, and anthracene and total aromatic metabolites in organs of coho salmon receiving the hydrocarbons by intraperitoneal injection. We examined the percentage of hydrocarbons and total metabolic products in animals over periods varying from 6 to 144 hr. The highest percentages of metabolites were found in the gall bladder at each time, regardless of which hydrocarbon was administered. The liver contained significant percentages of metabolic products at each time, irrespective of the hydrocarbon administered. Yet, the accumulation and retention of metabolic products was not restricted to the gall bladder and liver. In the case of naphthalene and anthracene-fed coho salmon, the 72- and 144-hr experiment demonstrated that significant amounts of metabolites were formed and retained in various parts of the body, such as brain, muscle, and "carcass." Data from the liver was notable in that the radioactivity associated with metabolites often exceeded 50% of the total radioactivity present. This finding applied, regardless of which aromatic hydrocarbon was administered.

In an attempt to delineate the specific structures of the metabolic products formed, we chromatographed solvent extracts of various tissues on thin layers of silicic acid employing new methods developed in our laboratories (Roubal et al. 1977b). The results from a 24-hr experiment, in which ^{14}C-labeled naphthalene was administered by intraperitoneal injection, revealed a wide spectrum of individual metabolites in the muscles, heart, gall bladder, and brain. In the brain, liver, and gall bladder, 1-naphthol and 1-naphthyl glucuronic acid comprised the highest percentage of the metabolites found; however, substantial amounts of glycoside/sulfate and mercapturic acid were also indicated. The heart and skeletal muscle were found to contain primarily 1-naphthol, 61 and 47%, respectively; however, in comparison to other organs, the heart contained appreciable amounts of the diol (26% of the extractable metabolic products). Generally speaking, the metabolites of naphthalene identified in this experiment were qualitatively similar to those found in exposed terrestrial animals (Terriere et al. 1971; Bakke and Scheline, 1970; LaDu et al. 1971).

PROBLEMS ENCOUNTERED

The only significant problem we have found is in obtaining highly pure radio-labeled aromatic hydrocarbons for studies on metabolite formation. We are addressing this difficulty in several ways, including employing special purification techniques, to resolve the problem in future studies.

RELEVANCE TO PROBLEMS OF PETROLEUM DEVELOPMENT

Data obtained thus far on the pelagic and demersal fish species show that these organisms are able to bioconcentrate water-soluble aromatic hydrocarbons in key tissues in short periods of time. The demersal species, starry flounder, appears to be particularly susceptible to accumulating complex mixtures of water-soluble petroleum hydrocarbons in most organs of the body. So pronounced is the tendency of the starry flounder to bioconcentrate certain alkyl-substituted naphthalenes from the water column that virtually imperceptible concentrations in the WSF become major components in key tissues, such as the liver. These findings point to the particular susceptibility of this species to accumulate aromatic hydrocarbons in proportions substantially exceeding those to which they are exposed in the marine environment. Put in another way, dermersal species may well be able to accumulate readily detectable levels of water-soluble aromatic hydrocarbons from marine environments that contain these compounds below the detectable limits of our most sophisticated analytical techniques. Moreover, it should be stressed that any attempt to relate point sources of petroleum pollution to hydrocarbon profiles in either the pelagic or demersal species examined, is severely hindered by rearrangements in concentrations of individual accumulated hydrocarbons arising through selective retention, metabolism, and excretion. Thus the comparison of hydrocarbon profiles from animals with profiles from the original oil, does not appear to be a valid method to monitor petroleum contamination.

The finding that both pelagic and demersal species retain aromatic hydrocarbons in direct relation to molecular weight and alkyl-substitution is worthy of comment. Demersal species, such as starry flounder, which appear to preferentially accumulate water-soluble aromatics associated with sediment, may be particularly susceptible to retaining relatively high proportions of alkyl-substituted benzenes and naphthalenes present in this system. Moreover, in terms of dietary exposure, both pelagic and demersal species may be expected to retain aromatic hydrocarbons of multiple ring structure for relatively long periods of time. The higher molecular weight aromatics might be presented to the organism (e.g., through the food-web) in association with particulate matter, through tar formation, or as micro-droplets. Thus, although physical and chemical aspects of the polluted environment may favor the evaporative weathering of the low molecular weight aromatic hydrocarbon fraction of crude oil, the remaining higher molecular weight structures may be of particular concern because of their longer retention time in the organisms.

We have accumulated a significant body of evidence to indicate that aromatic hydrocarbons are rapidly and progressively converted to metabolites in the pelagic and demersal fish studies and in invertebrate larval forms. Several cases have been documented in which the concentrations of metabolite exceed the concentrations of the parent hydrocarbons in tissues. The liver and skin have been identified as major sites for the preferential accumulation of metabolites over hydrocarbons in marine fish. In our studies thus far with marine fish and invertebrate larval forms, we have found no exception to the rule that hydrocarbons are readily depurated in clean seawater, whereas metabolites are retained for relatively long periods of time. Presently, we are not able to assess the possible toxic effects relating to the retention of metabolites. However, it seems evident that the oxidation of aromatic hydrocarbons to form mono- and di-hydroxy derivatives involves the formation of arene oxides as obligatory intermediates. We have no information at present on the concentration or residence time of arene oxides in tissues of the arctic and subarctic marine organisms. However, the literature on animal systems in general affirms the concept that extremely low levels of arene oxides are damaging to biological systems in many ways, which includes their strong mutagenic and carcinogenic potential. Certainly, the tendency of starry flounder to accumulate high proportions of metabolites in skin is of interest with respect to their known suceptibility to lesion formation in this tissue.

Evidence presented for the role of mucus in the excretion of naphthalene and metabolites in fish suggests that such exudates must be considered along with other routes of elimination which lead to the recycling of petroleum and metabolic products in the marine environment.

Our detailed studies on the site-specific accumulation of hydrocarbons and metabolites in marine organisms suggest that certain sites, such as skin, liver, dark muscle, gall bladder, and mucus of fish and thoracic segments of invertebrates, are primary targets for pinpointing petroleum pollution. These findings should be helpful in future chemical studies of hydrocarbon accumulations in marine organisms. Moreover, they emphasize that petroleum contaminants may be overlooked when whole animal tissues are used in field sampling procedures.

Generally, the findings to date imply that the pelagic species may be less suceptible to accumulating aromatic hydrocarbons from the environment than the demersal species, although a wider variety of different organisms must be studied in each class to verify this hypothesis. Clearly, the significant formation and retention of metabolites in tissues of petroleum exposed fish elicits a concern for possible deleterious biological effects on the resource, the magnitude of which should be evaluated through future research.

RECOMMENDATIONS FOR FUTURE RESEARCH

1. Studies on metabolite formation should be continued. Research should be directed toward formation and retention of metabolic products of high toxicity to animal systems, such as the arene oxides. Moreover, work is urgently needed to determine how long potentially toxic metabolites are retained in arctic marine organisms and whether the biological impact from these compounds is changed by transmission through the food web. The formation and retention of metabolites in marine organisms should be investigated in a complementary way with respect to alterations in biological systems, particularly in terms of chronic effects, such as neoplasia.

2. Ocean waters are a vast and complex pool of miscellaneous chemicals. Moreover, petroleum itself is made up of complex mixtures of hydrocarbons and metals and, in the marine environment, also is converted to hydrocarbon degradation products resulting from oxidative weathering. Little is known about the influences of such multiple chemical systems (synergism/antagonism) on the dispostion and metabolism of petroleum in marine biota. Despite the complications described, present evidence on the bioconversion of petroleum is based, almost exclusively, on experiments with single low molecular weight aromatic compounds. Thus, studies should be initiated to elucidate the influence of multiple-component systems on the dispositon and metabolism of petroleum in exposed arctic and subarctic organisms.

3. Very little is known about the biological fate of oxidatively weathered petroleum. It is time that studies were conducted to evaluate the extent to which marine organisms accumulate, metabolize, and excrete products of chemical and microbial oxidative processes.

4. Virtually no information exists on the bioaccumulation and metabolism of high molecular weight components (e.g., resinous materials) of petroleum, or, in fact, about aromatic compounds exceeding five benzenoid rings. Present techniques of high pressure liquid chromatography and complementary methods now make it possible to study the biological fate of these high molecular weight petroleum components. Relevant studies should be initiated in the future.

5. Little information is available on the disposition and metabolism of petroleum in marine organisms exposed to hydrocarbons associated in various ways with the new generation of dispersants. This gap in our understanding of the fate of emulsified petroleum is of considerable importance for the immediate future.

6. Preliminary evidence from our studies suggest that substantial differences in the biological fate of aromatic hydrocarbons are found in relation to whether the aromatic hydrocarbons are administered as single compounds or whether they are bound to macromolecules. Because aromatic

hydrocarbons are known to readily associate with a variety of macromolecules in aqueous systems (including dispersants), knowledge of the effects of such complexes on arctic and subarctic marine organisms is relevant and important. Preliminary studies in this area are recommended.

7. An immediate need exists to evaluate the potential for demersal organisms to accumulate and metabolize aromatic hydrocarbons when exposed to petroleum-impregnated sediments. The work which we have initiated with demersal species should be continued and extended to other bottom-dwelling species.

The above activities would provide a broad understanding of the biological fate of important petroleum components in the range of 1-5 benzenoid rings, as well as an initial understanding of the biological fate of high molecular weight compounds (e.g., resinous substances) and oxygenated components of petroleum, arising from biological and chemical degradations, which have been almost entirely neglected.

BIOLOGICAL FATE OF METALS IN FISH

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OBJECTIVES AND RATIONALE

Unlike the petroleum hydrocarbons, which are ultimately subjected to biodegradation, the metals are persistent in the environment. Only biological and chemical alterations take place, sometimes to toxic forms. Any assessment of the biological and biochemical effects of trace metals from petroleum on the marine biota must taken into account concentrations of these metals in petroleum, drilling chemicals, and production brines. Moreover, concentrations of the metals naturally present in the seawater, the chemical form and reactivity of metal in seawater are also of great importance (Clark, 1976). A wide spectrum of data exists suggesting that marine organisms are susceptible to detrimental physiological changes when exposed to trace metals (Varanasi and Malins, 1976; Reichert, 1976). However, very little is known about the chronic effects of trace metals on fish living under arctic conditions. Accordingly, we proposed to investigate uptake, distribution, metabolism, and excretion of metals in selected species present in areas involved in oil exploration.

Lead and cadmium have been chosen for investigation because: (a) They are known to occur in petroleum at trace levels and are toxic to some marine organisms at such levels (McKee and Wolf, 1963; Halstead, 1972; Clark, 1976); (b) most studies reported so far on uptake and accumulation of lead and cadmium in marine organisms have employed very high concentrations ($\sim$ppm) of these metals; and (c) bioconcentrations and physiological effects of lead and cadmium at the lower temperatures characteristic for arctic conditions have not been delineated.

To obtain the most comprehensive and in-depth evaluation of the impact of the trace metals on arctic and subarctic organsims, the investigation was carried out in three separate phases as follows: (A) Biochemical interactions of trace metals in fish. This study deals with patterns of uptake, accumulation, and retention of lead and cadmium in key tissues of fish exposed to low levels of water-borne metals at 4° and 10°C. Subcellular distribution of metals as well as mechanisms of excretion are also studied under this program. (B) Evaluation of importance of skin and epidermal mucus in metabolism and excretion of metals. This study is undertaken to define uptake, accumulation, and discharge of trace metals from epidermal mucus, skin, and scales of fish. Moreover, effect of metal exposure on mucus synthesis was also studied. (C) Influence of metal exposure on hydrocarbon metabolism in fish. This study was recently initiated to assess alterations in the profiles of metabolites of hydrocarbons (e.g., naphthalene) in tissues of fish exposed to lead and cadmium.

Coho salmon (Oncorhynchus kisutch) and starry flounder (Platichthys stellatus) were chosen as representative pelagic and demersal organisms, respectively, from subarctic and arctic regions. Fish were exposed at 3 and 150 ppb of the water-borne metals at 4° and 10°C. Because relatively low levels of metals were used in our experiments and also because the fish contained certain amounts of residual metals in most tissues, radioisotopes (^{210}Pb and ^{109}Cd) were used to provide a sensitive method of analyzing very low levels of the trace metals accumulated in tissues from metal exposures. In some experiments, lead and cadmium salts were intravenously injected in the test organisms because this mode of exposure bypasses all sites of uptake and insures that fish are exposed to a known amount of metals. This mode of exposure was used to study the subcellular distribution of radiolead or radiocadmium and also to study excretion of metals via epidermal mucus.

Only outlines of methods and results of these studies will be described. Some of the data are also combined together in tables to make the discussion concise. Details of these experiments are given in the OCSEAP Annual Report for RU 73/74 (1977) and in the publications emanating from this research.

METHODS

EXPOSURE REGIME

Water-immersion Studies

Saltwater-adapted coho salmon (200 ± 20 g) and starry flounder (30 ± 15 g) were acclimatized at experimental temperature (4° or 10°C) for a period of two weeks and exposed to either water-borne lead and cadmium (150 ppb) under partially flow-through conditions as described previously (Varanasi and Markey, 1977).

The fish were exposed to either lead or cadmium for a period of two weeks at each temperature and samples were taken from three or four fish at the 4th, 9th, and 14th day. At the end of the two-week period, remaining fish were placed in control water for depuration of tissues and samples were taken on 7th and 37th day. A group of control fish of similar weight was kept under identical conditions in seawater. Concentrations of lead and cadmium in control seawater were less than 5 and 2 ppb, respectively.

Injection Studies

In separate experiments, coho salmon (80 ± 5 g) in salt water were injected intravenously with 32± 2 μg of metals as either lead nitrate mixed with ^{210}Pb or cadmium chloride mixed with ^{109}Cd dissolved in 250 μl of tris buffer (pH 7.2) as described previously (Varanasi and Markey, 1977; Reichert et al. 1977). A similar experiment was carried out with a group of fish which was exposed to 200 ppb of cold cadmium or lead for two weeks prior to intravenous injection to determine if prior exposure to metals caused any alterations in radioactivity associated with metal binding proteins.

Metal/Hydrocarbon Exposure

Coho salmon were exposed to 200 ppb of either lead or cadmium in water for three weeks and then fed 334 ppm of aromatic hydrocarbon mixture containing equal amounts of phenanthrene, 2-methylnaphthalene and 2,6-dimethylnaphthalene. Control fish were also fed this hydrocarbon mixture. A week later, both groups were injected intravenously with ^{14}C-labeled naphthalene. Twenty-four hours after the injection fish were analyzed (Reichert, Frederighi, Thomas, and Malins, unpublished).

ANALYTICAL METHODS

Samples of mucus, intact skin, scales, skin, blood, gills, liver, and kidney were obtained (Varanasi and Gmur, 1977) and concentrations of ^{210}Pb or ^{109}Cd were determined by liquid scintillation spectrometry as described previously (Varanasi and Markey, 1977; Reichert et al. 1977). Concentrations of total Pb or Cd in tissues, expressed on a wet weight basis, were obtained from ratios of $^{210}Pb/Pb$ and $^{109}Cd/Cd$ in standard solutions. Concentrations of cold lead and cadmium in seawater and fish tissues were determined by Laucks Analytical Laboratories (Seattle, Washington).

Samples of liver and kidney of fish from the injection study were homogenized and cytosol was obtained according to the method of Chen et al. (1974). These samples were fractionated on a column packed with Sephadex G-75 superfine. The radioactivity in the eluted fractions was determined to assess distribution of metals bound to various protein fractions. The protein concentration in each eluant was determined by a modified method of Lowry et al. (1951).

Analyses of the individual metabolites of naphthalene in liver and gall bladder of the metal-exposed and control fish (both groups were injected with radioactively-labeled naphthalene) were carried out by Dr. Larry Thomas of National Analytical Facility using high pressure liquid chromatography.

RESULTS

EFFECTS OF TEMPERATURE ON BIOCONCENTRATION OF METALS IN FISH

Our findings (Varanasi and Markey, 1977; Reichert et al. 1977) indicated that low (ppb) levels of lead and cadmium in seawater can be translated into ppm levels in tissues of both coho salmon and starry flounder (bioconcentration values given in Tables 1 and 2 are based on

TABLE 1. *Effect of temperature on bioconcentration*[a] *of lead and cadmium in tissues of saltwater-adapted coho salmon (Oncorhynchus kisutch) exposed to water-borne metals*[b]

Metal	Temp. °C	Mucus	Scales[c]	Liver	Brain	Kidney[d]	
						a	p
Pb	4	1.5	10.0	1.8	ND[e]	1.8	7.3
	10	1.8	24.9	3.0	ND	3.1	12.0
Cd	4	1.0	0.3	1.1	ND	0.7	0.9
	10	1.2	0.7	2.0	ND	1.2	2.5

[a] *Bioconcentration=metal concentration in tissue (ng/g, wet wt)/metal concentration in seawater (ng/ml).*
[b] *Fish were exposed to 150 ppb of either lead or cadmium for two weeks.*
[c] *Scales with dermal and epidermal cells attached.*
[d] *a=anterior kidney; p=posterior kidney.*
[e] *ND=not detected.*

TABLE 2. *Effect of temperature on bioconcentration*[a] *of lead and cadmium in tissues of starry flounder (Platichthys stellatus) exposed to water-borne metals*[b]

Metal	Temp. °C	Mucus	Skin[c]	Liver	Brain	Posterior kidney
Pb	4	4.2	1.2	3.9	1.3	5.7
	10	2.1	2.4	5.0	3.5	8.6
Cd	4	1.3	0.3	3.0	ND[d]	0.5
	10	1.3	1.1	10.5	ND	1.9

[a] *Bioconcentration=Pb concentration in tissue (wet wt)/Pb concentration in water.*
[b] *Fish were exposed to 150 ppb of either lead or cadmium for two weeks.*
[c] *Skin with scales.*
[d] *ND=not detected.*

wet weight of tissues). These results should be considered together with the fact that starry flounder, a bottom-dweller, may be exposed to additional influx of metals present in the sediment. Data in Table 2 show that high concentrations of lead accumulated in the brain of starry flounder. Since lead is a neurotoxin, high levels of lead in starry flounder brain suggest possible neurological damage that could have behavioral consequences. Another notable finding was that both species accumulate substantial concentrations of lead and cadmium in kidney, especially in the posterior section. Concentrations of metal in epidermal mucus of coho salmon were slightly greater than the levels in the surrounding water; however, metals (especially lead) were bioconcentrated to a much greater extent in starry flounder mucus. These findings together with our previous results on rainbow trout mucus (Varanasi et al. 1975) and our studies with freshwater coho salmon indicate that levels of metals accumulated in epidermal mucus are dependent largely on a particular species of fish rather than the salinity of the medium. In view of the fact that epidermal mucus plays an important role in both defense and protective mechanisms in fish, high concentrations of metals sequestered in starry flounder mucus may have deleterious consequences.

The results in Table 1 and 2 show that the temperature of the surrounding water had definite influence on the uptake and accumulation of metals in both fish species under investigation. All tissues, e.g., skin, scales, gills, blood, liver, and kidney, of fish at 10°C had considerably higher metal concentrations than the tissues of fish at 4°C (Tables 1, 2). It is evident from these results tht fish living under arctic temperatures may accumulate relatively smaller concentrations of metals. However, the effect of low temperature on turnover and retention of metals should also be taken into account when considering overall effect of trace metals on arctic biota.

RETENTION OF METALS IN KEY TISSUES

Temperature of the surrounding water appears to have distinct effect on retention of metals in tissues of starry flounder. For example, after 7 days of depuration, fish maintained at 10°C retained much lower concentrations of lead in tissues such as skin, gills, brain, and kidney compared to the lead concentrations in tissues of fish at 4°C (Fig. 1). No such temperature effect was observed for coho salmon.

When the metal-exposed coho salmon were placed for several days in seawater, free of radioactive metals, lead concentrations increased in most tissues but decreased in blood indicating transfer of lead from the blood to other tissues. Cadmium concentrations, on the other hand, decreased significantly in all tissues except the kidney of cadmium-exposed coho salmon after several days of depuration. For example, liver, scales, posterior kidney, and blood of lead-exposed coho salmon contained 109, 114, 110, and 74%, respectively, of the lead concentrations accumulated in these tissues at the end of the two-week exposure at 10°C. These same tissues of cadmium-exposed fish contained 87, 53,

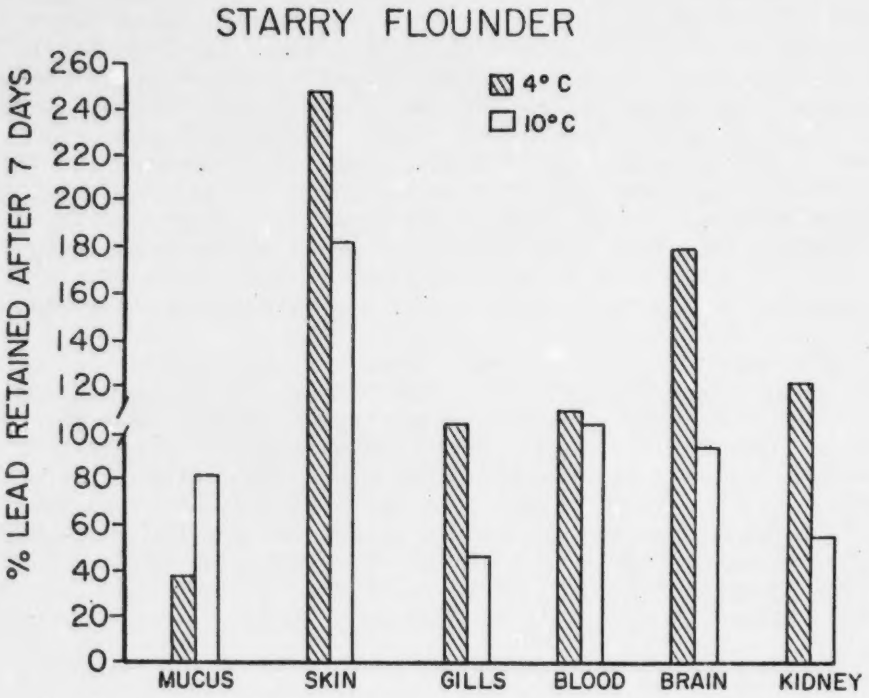

Figure 1. Effect of temperature on lead concentrations retained in the tissues of lead-exposed starry flounder after one week of depuration.

156, and 79%, respectively, of the original cadmium concentrations (Varanasi and Markey, 1977, Reichert et al. 1977). Lead was also retained to a much greater extent than cadmium in tissues of starry flounder after a 7-day depuration.

After 37 days of depuration, substantial (>50%) concentrations of both lead and cadmium still persisted in most tissues of coho salmon. Because of mortality, depuration of starry flounder tissues was not studied for periods longer than 7 days. A notable finding was that metal concentrations in kidney of coho salmon continued to increase even after the fish were placed in seawater for a period of five weeks. Thus, termination of exposures to metals should not be interpreted to mean that metal concentrations will necessarily remain constant or decline in the kidney and possibly other sites. It is interesting to note that in cases where a decline in cadmium or lead concentrations was observed, e.g., in gills, significant levels still remained even after five weeks in control seawater. The strong tendency to sequester cadmium and lead in gills (Reichert et al. 1977) during uptake and depuration periods raises questions about possible interference with osmoregulation and oxygen consumption (Thurberg et al. 1973).

Another important finding was that more than 50% of lead and cadmium accumulated in the scales of coho salmon was still present after five weeks of depuration (Varanasi and Markey 1977). It is known that in salmonids skin and scales play an important role in calcium transport and metabolism of fish (Podoliak and Holden, 1964). The persistence of high concentrations of lead and smaller but significant concentrations of cadmium may interfere with transport and perhaps metabolism of calcium in fish. Moreover, scales of salmonids are known to be resorbed during maturation and the mineral components of the scales are utilized for general metabolism and production of sexual components (Wallin, 1957). In the metal-exposed fish, resorption of scales may result in the release of toxic metals in the bloodstream at the time of stress. The consequences of release of such toxic metals on eggs and sperm remains to be assessed. It should be noted with regard to these findings that recent studies from our laboratories (Varanasi and Gmur, 1977) have shown that increased levels of environmental or dietary calcium significantly reduces uptake and retention, and presumably toxicity, of lead in coho salmon.

DISTRIBUTION OF CADMIUM AND LEAD IN SUBCELLULAR FRACTIONS

The findings (Reichert et al. (1977) demonstrating the tendency to bioconcentrate cadmium and lead in vital organs such as liver and kidney of both coho salmon and starry flounder warranted further studies to investigate subcellular distribution of these metals. The results show that cadmium tends to accumulate in portions of cells mediating vital biochemical and physiological processes, e.g., cytosol. For example, cytosol fractions of liver and posterior kidney of cadmium-exposed starry flounder contained 72 and 80% of total cadmium (^{109}Cd), respectively, and cytosol fractions of these tissues from coho salmon contained 64 and 56% of total cadmium (^{109}Cd), respectively. Microsomal fractions rich in enzyme systems also contained significant concentrations of cadmium. Distribution of lead was somewhat different, liver of lead-exposed coho salmon contained almost equal proportions of lead in mitochondria, microsomes, and cytosol. It is demonstrated in mammalian systems that lead accumulates in the form of intranuclear inclusion bodies containing a complex of lead, calcium, and phosphorus (Carroll et al. 1970).

Cadmium has a tendency to compete with zinc for binding sites on metalloproteins of liver and kidney in mammalian systems, which is well documented (Vallee and Ulmer, 1972). Very little information is available on cadmium-binding proteins (CdBP) in fish. Accordingly, radiotracer techniques using intravenous injection of ^{109}Cd were employed to assess if cadmium in gills, liver, and kidney of cadmium-exposed fish was bound to metalloproteins (M.W.~10,000 daltons). Moreover, in mammalian systems, it is demonstrated that injection or injestion of cadmium stimulates synthesis of CdBP in liver presumably to provide protection against toxic effects of cadmium (Nordberg, 1972; Sabbioni and Marafante, 1975). Results with coho salmon (Reichert et al. 1977) show that similar to mammalian systems, a major fraction of the ^{109}Cd was bound to CdBP$\underset{\sim}{}$8,900) in liver and kidney of coho salmon. Moreover, the percentage of the total ^{109}Cd in cytosol bound to CdBP was greater in those fish which had been exposed to 200 ppb Cd in seawater for two weeks, compared to controls in seawater only (Table 3). The results imply that prior exposure to cadmium induces synthesis of CdBP in key tissues of fish. It appears from these results that in salmonids, a detoxification mechanism similar to mammalian systems may exist to provide protection against toxic effects of cadmium. Further studies are necessary to investigate whether other fish species (e.g., starry flounder) are able to bind and detoxify metals such as cadmium.

EFFECT OF METAL EXPOSURE ON SYNTHESIS OF EPIDERMAL MUCUS

Epidermal mucus plays an important role in defense and protective mechanisms in fish; therefore, the effects of metal exposure on synthesis of epidermal mucus were studied. Moreover, the ability of mucus to sequester metals from surrounding water as well as discharge of metals from the mucus of the metal-exposed fish was also investigated.

To assess the effect of water-borne metals on mucus production, weight of mucus was determined for each test and control coho salmon throughout the experiment. Values for weight of mucus per gram of fish were obtained for 10 fish in each experiment (Table 4). The results in Table 4 show that at 10°C, 150 ppb of either lead or cadmium in surrounding water induced significantly higher ($P<0.05$) mucus production in coho salmon (Varanasi and Markey, 1977).

TABLE 3. *Distribution of Cd-109 in coho salmon cytosol from gill and liver at 3 and 48 hours post injection*[a]

Sample	Exposure	Sampling	Cd bound to high MW fractions	Cd bound to CDBP fractions[c]	Cd bound to remaining fractions	Cd in cytosol
			ng ^{109}Cd/mg cytosolic protein			
Gills	Seawater only[b]	3	16	2.0	3.7	21.7
		48	293	19.0	14.0	57.3
	200 ppb Cd	3	9.5	16.3	3.8	29.6
Liver	Seawater only	3	12.6	67.5	5.2	85.3
		48	24.3	167	8.5	200
	200 ppb Cd	3	5.6	73.8	3.1	82.5

[a] *Fish were maintained at 10°C during exposures to cadmium-109.*
[b] *Fish were exposed to 200 ppb cadmium for 2 weeks prior to injection of cadmium-109.*
[c] *CdBP (MW≃8900).*

Table 4. Average weight of epidermal mucus obtained from control and metal exposed fish[a]

Sample	Metal	Mg mucus/g fish
Control[b]	--	7.2 ± 1.6 (10)[a]
Test	Pb	12.7 ± 2.8 (10)[c]
Test	Cd	12.1 ± 2.9 (10)[c]

[a] Fish were exposed to 150 ppb of either lead of cadmium for a period of two weeks at 10°C.

[b] Control seawater contained 5 ppb of lead and 2 ppb of cadmium.

[c] Significantly different ($P<0.05$).

These results show that short-term exposure to pollutants triggers increases synthesis of mucus; however, consequences of long-term exposure on mucus production remains to be assessed. Sherwood and Bendele (1975) reported that in Dover sole collected from Palos Verdes, a known area of contamination (PCB, Cr, Cd), fin erosion was accompanied by reduction in epidermal mucus (visual observation). Whether or not there exists a relationship between altered rate of mucus production and pathological surface conditions is not known.

Our results (Varanasi and Markey, 1977) show that when the fish were injected with either lead (^{210}Pb) or cadmium (^{109}Cd) salts substantial concentrations of lead (164 ppb, dry wt) and cadmium (744 ppb, dry wt) were present in the mucus for the few days following the injection. This finding together with the results of the water-immersion study showing that a significant fraction of the accumulated metals was persistent in the mucus of the test fish for several days during depuration suggests that metals are discharged in epithelial mucin via the mucous cells and excreted in the mucus. In view of the fact (Pickering, 1976) that epidermal mucus of salmonids exists in a state of continuous flux (that is, small amounts of mucus are continuously sloughed off and renewed), our findings demonstrate, for the first time, that epidermal mucus may play an important role in excretion of metals (Varanasi and Markey, 1977). An intriguing possibility exists that increased production (or increased turnover rate) of epidermal mucus in the challenged fish is related to the role of the mucus in discharge of the pollutant. That is, it may be that a certain critical concentration of a pollutant in blood at a given temperature may trigger increased synthesis of epithelial mucin resulting in rapid turnover of mucus which, in turn, would expedite discharge of the pollutant via epidermal mucus. With respect to this hypothesis, it should be noted that naphthalene and its metabolic products are also shown to be released in epidermal mucus of naphthalene-exposed rainbow trout (Varanasi et al. 1977).

POSSIBLE EFFECT OF METAL-EXPOSURE ON HYDROCARBON METABOLISM

A test group of coho salmon were exposed to 200 ppb lead for three weeks at 10°C. Both control and lead-exposed coho salmon were then fed Oregon moist pellets (OMP) containing 334 ppm of aromatic hydrocarbons one week before intravenous injection of ^{14}C-naphthalene. Results show that lead-exposed fish contained 70 to 80% ($P<0.05$) higher amount of the metabolite, ^{14}C-1,2-dihydro-1,2,-dihydroxynaphthalene, in the liver as compared to the amount of this metabolite in the control livers (Reichert et al., unpublished).

It appears from these preliminary results that exposure to low levels of lead causes alteration in ability of fish to metabolize and/or excrete metabolites of hydrocarbons. The findings imply that lead and presumably other divalent metals may possibly alter the disposition of petroleum aromatic hydrocarbons in marine fish.

RELEVANCE TO PROBLEMS OF PETROLEUM DEVELOPMENT

1. Salmon and starry flounder readily accumulate lead and cadmium from seawater. The metals in liver and kidney are stored to a substantial degree in portions of cells mediating vital biochemical and physiological processes (e.g., cytosol). Cadmium is preferentially bound by low molecular weight proteins ($\simeq$9,000 daltons) but is also associated with high molecular weight proteins of the cytosol. Lead shows a strong preference for neural tissue (e.g., brain). The findings imply that low concentrations of metals entering marine water through petroleum drilling and transport operations are likely to significantly increase the metal burden of key tissues of fish and thereby possibly alter normal physiological processes.

2. Decrease in environmental temperature from 10° to 4°C reduced accumulation of metals in fish tissue by almost 50%. Hence, it would appear that under arctic conditions of low temperature, fish will accumulate considerably lower concentrations of metals. However, in starry flounder, low temperature also caused slower discharge or turnover or metals indicating longer retention time for metals in tissues of fish living under arctic conditions.

3. Epidermal mucus of coho salmon is involved to some extent in excretion of lead and cadmium. The importance of this route in relation to other excretory tissue (e.g., kidney) remains to be assessed. Exposure to sublethal levels (150 ppb) of lead and cadmium for periods of up to two weeks resulted in increased production ($\simeq$40% increase) of epidermal mucus in coho salmon. This increased rate of production may induce alterations in physicochemical properties of mucus. Effect of long-term metal exposure on mucus production remains to be assessed and whether there exists a possible relation between altered turnover rate of epidermal mucus and pathological conditions of skin surface is not known at present.

4. Our results also show that skin and scales act as storage and perhaps detoxification sites for metals in both salmonids and flatfish. Substantial amounts of metals persisted in the skin and scales several weeks (37 days) after fish were returned to clean water. It is anticipated that persistence of high concentrations of toxic metals would have adverse effects on skin structure; moreover, what effect resorption of scales of the metal-exposed fish would have on viability of eggs and sperm is not known.

5. The results show that exposure to lead alters metabolism and disposition of petroleum hydrocarbons in marine fish.

PROBLEMS ENCOUNTERED

There were no major problems encountered which prevented completion of the main theme of the proposed research.

RECOMMENDATIONS FOR FUTURE RESEARCH

From the results obtained so far, we feel that the following studies should be carried out in the future to understand more clearly the impact of metals on arctic and subarctic marine organisms.

1. Study on possible effects of metal-exposure on hydrocarbon metabolism should be continued using several different species of fish.

2. Studies to investigate the effect of metals (lead and cadmium) on calcium metabolism in fish should yield much needed information on how metals affect important biochemical processes in arctic and subarctic organisms.

3. Our results show that salmonids and flatfish accumulate substantial amounts of lead in the skin. Lead stored in salmonid skin is primarily associated with the scales. Resorption of scales occurs in salmonids during maturation for utilization of minerals for general metabolism and production of sexual components (Wallin, 1957). This known process of mineral (calcium) mobilization from scales raises questions about the biological consequences of lead stored in scales on developing eggs and sperm. Studies should be conducted to investigate transport of lead from the scales to eggs and sperm and the effect of this lead on their viability.

4. Heavy metals have been shown to influence the development and hatching of eggs of marine fish. At present, virtually no information is available on possible synergistic effects of aromatic hydrocarbons and metals on hatching and development of eggs. The proposed study would determine possible interactive effects of petroleum hydrocarbons and heavy metals (lead and cadmium) on the development of marine fish

eggs, specifically of starry flounder. In addition, studies to determine whether heavy metals alter the activity of the hatching enzymes; namely, chorionases should be of interest.

THE EFFECTS OF ALASKAN CRUDE OIL ON FLATFISH, AND THE PREVALENCE OF FISH PATHOLOGY IN ALASKAN MARINE WATERS

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OBJECTIVES AND RATIONALE

The nature and potential severity of pathological effects resulting from long-term exposure of marine fish to chronic levels of Alaskan crude oil are poorly understood. Certain polynuclear aromatic hydrocarbons (PAHs) which are found in Alaskan crude oil, are known to cause pathological conditions in mammals and fish either directly by the action of toxic components and carcinogens (Hueper and Payne, 1960; Bingham et al. 1965; Sullivan, 1974), or indirectly by inhibiting disease resistance mechanisms which normally protect an animal from microbial infections (Stjernswäard, 1974; Kripka and Weiss, 1970). In order to recognize pathological conditions of feral marine fishes resulting from pollution of Alaskan waters with crude oil, information on baseline levels of pathological conditions in these fish populations must be available. This report presents data from two separate studies funded by separate Outer Continental Shelf Environmental Assessment Program (OCSEAP) contracts. One study concerns the longterm pathological effects of exposing flatfish to sediments contaminated with Alaskan crude oil (OCSEAP Research Unit 73). The other study deals with the prevalence, distribution, and pathology of disease of demersal fishes in the Bering Sea, Gulf of Alaska, Norton Sound, and Chukchi Sea (OCSEAP Research Unit 332).

The pathological effects of crude oil-contaminated sediments on flatfish were investigated by a multidisciplinary effort involving the fields of pathology, analytical chemistry, fishery biology, and electron microscopy. Levels of petroleum hydrocarbons in the sediment and fish were monitored, and fish were periodically examined for histological, hematological, gross anatomical, ultrastructural, and behavioral changes (McCain et al. 1978). Flatfish were selected as experimental animals because (1) they live in close association with bottom sediments which may become heavily contaminated with petroleum hydrocarbons as a result of oil spills (Blumer and Sass, 1972), (2) flatfish are known to bioconcentrate high levels of petroleum hydrocarbons (Roubal et al. 1977), and (3) over a dozen species of flatfish in the Pacific Ocean have been reported to have skin papillomas (tumors) of as yet unknown etiology (Wellings et al. 1976).

In addition to identifying the types of pathological conditions in Alaskan marine fish and their prevalence and geographical distribution, the baseline study included efforts to determine the cause(s) of these conditions (McCain et al. 1977b, Alpers et al. 1977, Wellings et al. 1977; McArn et al. 1978). Knowledge of the possible causes may provide a clearer understanding of the ways in which exposure of an animal to oil could affect the prevalence of certain abnormalities.

METHODS

EFFECTS OF ALASKAN CRUDE OIL

Sediment was collected from a beach near Sequim, Washington. It had no detectable aromatic hydrocarbons, and contained iron, copper, and zinc in concentrations of 1,210, 11.5, and 9.0 mg/kg (dry wt), respectively. Particle-size analyses showed that 87% of the sediment particles were between 0.18 and 0.84 mm in diameter. A 0.2% (v/v) mixture of Alaskan crude oil and sediment was prepared using a cement mixer. The sediment was placed in a specially constructed aquarium (Fig. 1) to a depth of 5 cm. After rinsing the sediment overnight by allowing seawater to flow through the tank, the experimental fish were added. An equal amount of uncontaminated sediment placed in a similarly designed aquarium at the same depth was used for control fish. Thirty-five English sole were placed in each aquarium.

After adding the fish to the sediment-containing aquaria, the aquaria were examined daily for: dead or moribund fish, the number of fish lying uncovered on the surface of the sediment, and for the general level of activity. The temperature ranged from 7° to 9°C with an average of approximately 8.5°C, and the average salinity was about 27 ppt.

Hydrocarbon analyses of sediment and fish tissue were performed by the NOAA National Analytical Facility using a modification of procedures described by MacLeod et al. (1976) which involved gravimetric determination of total extractable hydrocarbons and gas-liquid chromatography (GLC) for alkane and arenes. By subtracting the concentration of total extractable hydrocarbons in the control sediment from the level of extractable hydrocarbons in the oil-contaminated sediment, the value of total extractable petroleum hydrocarbons (TEPH) was determined. Samples of oil-contaminated sediments (about 400 g/sample) were taken immediately after mixing, and at 2, 7, 16, 30, 51, and 148 days after being placed in the aquarium.

At 11 days and at approximately 1, 2, 3, and 4 months of exposure to the sediments, 3 fish from each group were sacrificed and tissue samples were subjected to histological, morphological, hematological, and chemical analyses; and the length and weight of all the fish were measured. For histological examination of each fish, pieces of gill, skin gastrointestinal tract, kidney, liver, and eye lense were removed and preserved in appropriate fixatives. Specimens to be examined by light microsocpy were embedded in paraffin, sectioned, and stained by a variety of histochemical stains and methods. Procedure used for electron microscopic examinations of tissues are described elsewhere (see Morphology section by J. W. Hawkes).

Hematological tests performed included the following: hemoglobin concentrations by the cyanomethemoglobin method, hematocrit, total red blood cell and leucocyte counts, and differential leucocyte counts.

Samples of muscle, skin, and liver of the fish were analyzed for petroleum hydrocarbons by GLC (MacLeod et al. 1976). Muscle samples from each fish were analyzed individually, and skin and liver samples from the three fish in each of the two groups were pooled. Tissue samples were frozen at -20°C immediately after they were taken and thawed just before chemical analysis.

BASELINE FISH DISEASE STUDIES

Demersal fishes were captured with an otter trawl operated either by the NOAA ship *Miller Freeman* or by the Polish research ship *Professor Siedlecki* in the Bering and Chukchi Seas, Norton Sound, and Gulf of Alaska (GOA). Gear characteristics, trawling methods, and sampling procedures were similar to those used by Kaimmer et al. (1976). Each catch was sorted according to species and a randomly-selected subsample of each species (200 to 400 fish) was used to estimate size, age composition, and sex ratios. The samples were examined for externally-visible pathological conditions and, when feasible, for readily recognizable internal disorders.

The types and frequencies of each abnormality found during these examinations were recorded. The species, sex, length, weight, and age of each affected fish were determined; and the type, location, and size of the pathological condition(s) was recorded. Either the diseased tissue alone, or the affected tissue in combination with the major internal organs, was preserved in 10% formalin with phosphate-buffered saline and returned to the laboratory and examined histologically by previously mentioned procedures. Isolates of bacteria, fungi, and virus were obtained by standard microbiological procedures.

RESULTS

EFFECTS OF ALASKAN CRUDE OIL

Approximately 40% of the total extractable petroleum hydrocarbons (TEPH) on the surface of the crude oil-contaminated sediment was released during the first 30 days of the experiment. The decrease during this period was from about 700 to 400 μg/g (dry wt). After this initial decrease, the concentration of TEPH in the surface of the sediment remained at about 400 μg/g (dry wt) for the remainder of the experiment. The most abundant aromatic hydrocarbons and their concentrations in the upper sediment during the first 30 days are presented in Figure 2.

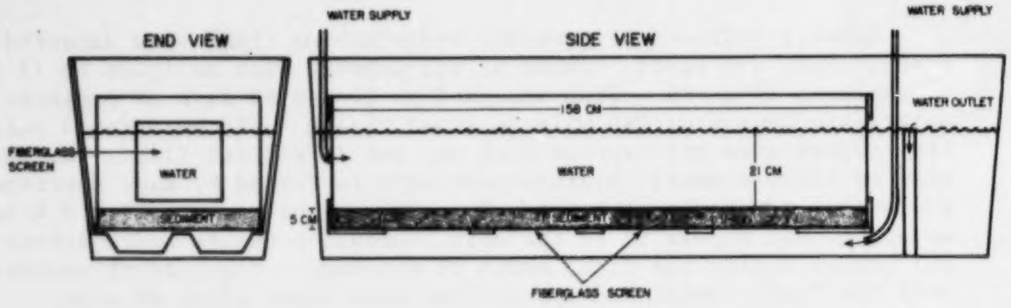

Figure 1. Diagram of aquaria used in experiments involving exposure of flatfish to oil-contaminated sediments.

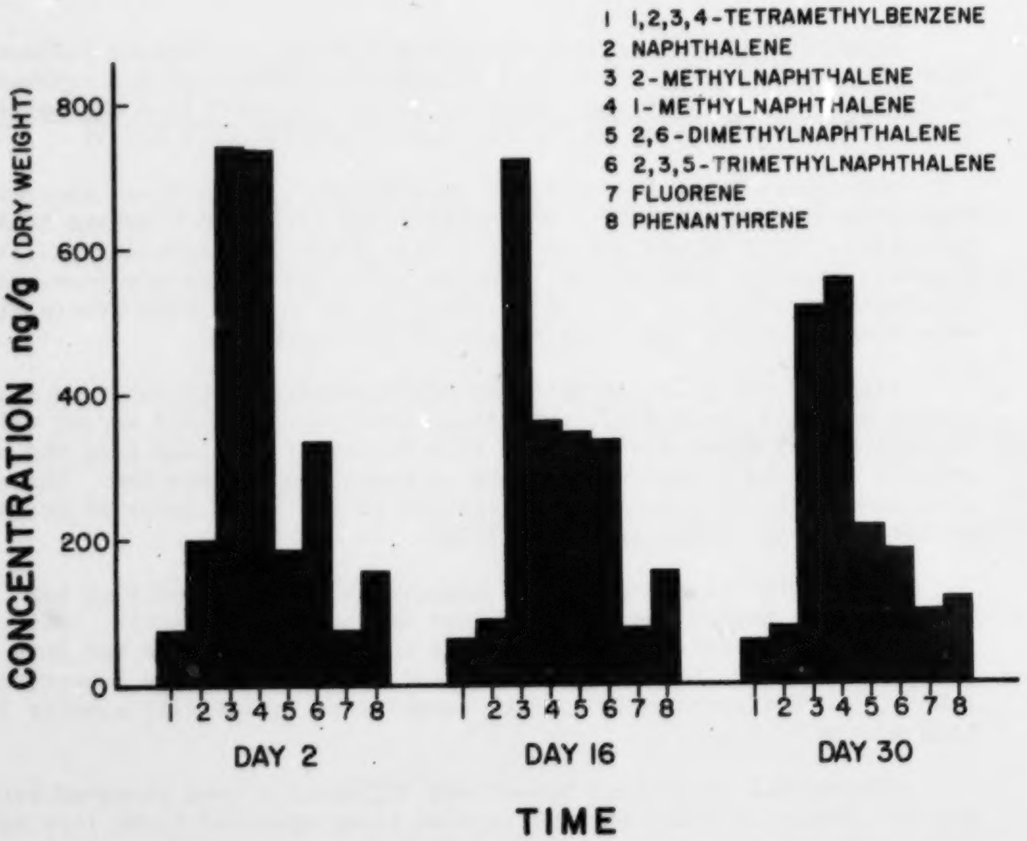

Figure 2. The concentrations of the 8 most abundant aromatic hydrocarbons in oil-contaminated sediments after 2, 16, and 30 days in the flow-through seawater aquaria.

Several polynuclear aromatic hydrocarbons (PAH) were detected in liver, skin, and muscle tissue of oil-exposed fish analyzed at 11 days of exposure (Fig. 3). Fish analyzed at 27 and 60 days of exposure had detectable levels of PAH only in liver tissue. PAH were found only in the tissues from oil-exposed fish and not in control fish. The identities of these aromatic hydrocarbons were confirmed by mass spectrometric analyses. 1-Methylnaphthalene, 2-methylnaphthalene, and 1,2,3,4-tetramethylbenzene appear to be the most abundant aromatic hydrocarbons in the livers during the first month of exposure. Significant amounts of only the latter benzenoid hydrocarbon were found after 60 days.

Both groups of fish lost weight during the first 2 mo of the experiment, with the oil-exposed group losing more than the controls (Fig. 4). After this period, the control fish began to regain weight, and by 4 mo 27% of the controls weighed less than their average weight at 2 weeks. the oil-exposed group was slower in their recovery from this initial weight loss; thus 69% of these fish weighed less than their earlier average weight by 4 mo. This weight difference was significant at the 95% confidence level.

Examination by light and electron microscopy of tissues collected from oil-exposed and control fish showed the presence of oil-related tissue abnormalities in the liver and gills. Tissues from the spleen, kidney, intestine, fins, of both groups were relatively normal.

Oil-exposed fish had a higher frequency of severe liver abnormalities than did control fish. Both groups had slight to moderate hepatocellular lipid vacuolization (HLV) (see Morphology section by J. W. Hawkes); however, only the oil-exposed group had the severe form, with a frequency of 17% (5 of 30 fish). Three of the 5 fish with severe HLV were sampled during the first months of oil exposure.

Although the gills of both the oil-exposed and control fish displayed some histopathology, including focal necrosis, and mucous cell and epithelial hyperplasia of the gill filaments and lamellae; the oil-exposed fish had a higher incidence of parasitic infestation. The most prominent forms of parasitic infestation of the gills involved trichodinids, microsporidia, and flagellates.

During the first 2 mo of the experiment, oil-exposed fish had significantly higher hematocrit values and hemoglobin levels. After 3 mo, however, the values of these tests were about the same for both groups. The other hematological parameters (total RBC and leucocyte counts, and differential leucocyte count) were essentially similar for both groups.

Slight, but consistent behavioral differences were observed between the two groups of fish, the oil-exposed group appeared to be less active (i.e., the average of the number of fish out of the sediment in the control aquarium per day was higher than that of the test aquarium).

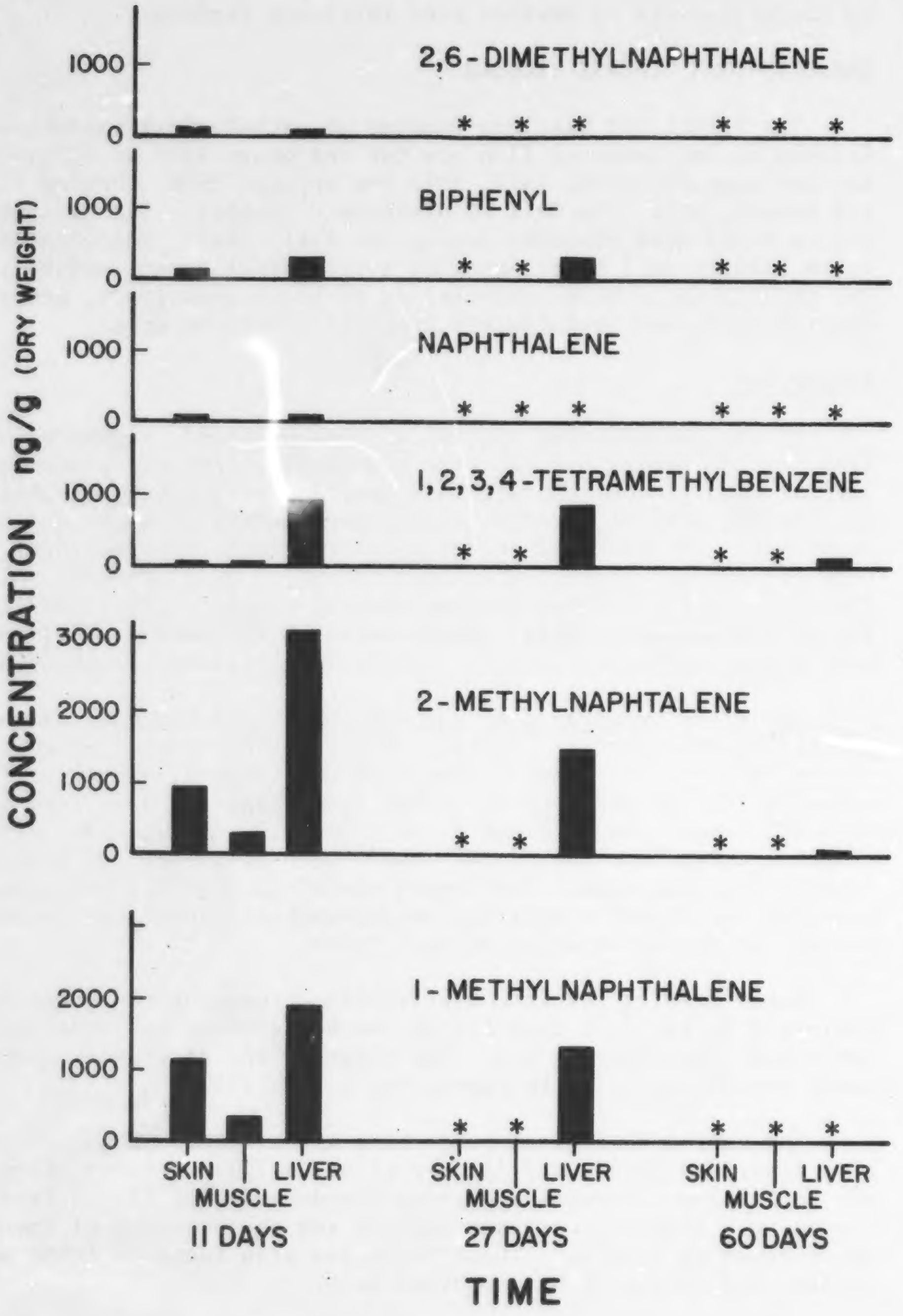

Figure 3. The levels of aromatic hydrocarbons in the tissues of English sole exposed to oil-contamined sediment for various times (*indicates not detected).

Also, the oil-exposed group were less active feeders, as was suggested by larger amounts of uneaten food following feeding.

BASELINE FISH DISEASE STUDIES

The results of baseline studies of pathological conditions in Alaskan marine demersal fish are for the years 1975 to 1977. The Bering Sea was sampled in the fall, 1975 and spring, 1976. During the winter and summer, 1977, the Gulf of Alaska was sampled. The Chukchi Sea and Norton Sound were examined during the fall, 1976. The presentation of these results will be arranged by geographical areas; and the pathological conditions, a brief description of their prevalence, geographical distribution, and pathological properties will be given.

Bering Sea

Of the 26 different species of fish examined, representing 67,000 fish, in the Bering Sea, 22 were essentially free of recognizable pathological conditions. The affected species, associated pathological conditions, and the overall average frequencies of each condition were as follows: Pacific cod (Gadus macrocephalus), pseudobranchial tumors, 8.3% (514/6164); Pacific cod, skin ulcers, 1.6% (73/4654); pollock (Theragra chalcogramma), pseudobranchial tumors, 1.7% (156/9173); yellowfin sole (Limanda aspera), lymphocystis, 2.4% (500/21,275); and rock sole (Lepidopsetta bilineata), epidermal papillomas, 1.1% (153/13,377).

The gross appearance of the cod tumors was the same as that described by McCain et al. (1977). Briefly, they ranged in color from yellow, to pink, to brown. They were oval-shaped, smooth-surfaced, extended into the pharyngeal cavity, and ranged in size from just larger than the normal pseudobranch to 50 x 30 x 20 mm (Fig. 5). With one possible exception, all of the tumors were bilateral and both were usually the same size. The tumors often had necrotic areas on the surface, and normal-appearing pseudobranchial tissue was located on the surface or in the interior of each tumor.

Tumor-bearing cod were distributed throughout the sampling area as indicated by the fact that 67% of the hauls where cod were captured contained tumor-bearing cod. The range of the disease frequencies in hauls containing cod with tumors was 1.1 to 100%.

The histopathological properties of the pseudobranchial tumors have been described previously (Alpers et al. 1977). Briefly, they included the separation of normal-appearing pseudobranchial tissue from the tumor tissue by a connective tissue capsule and the presence of tumor-specific cells known as X-cells. These cells are also found in other marine fish tumors, and they will be discussed later.

The pollack pseudobranchial tumors and the cod tumors were usually grossly similar in color, shape, and texture (Fig. 6). However, the

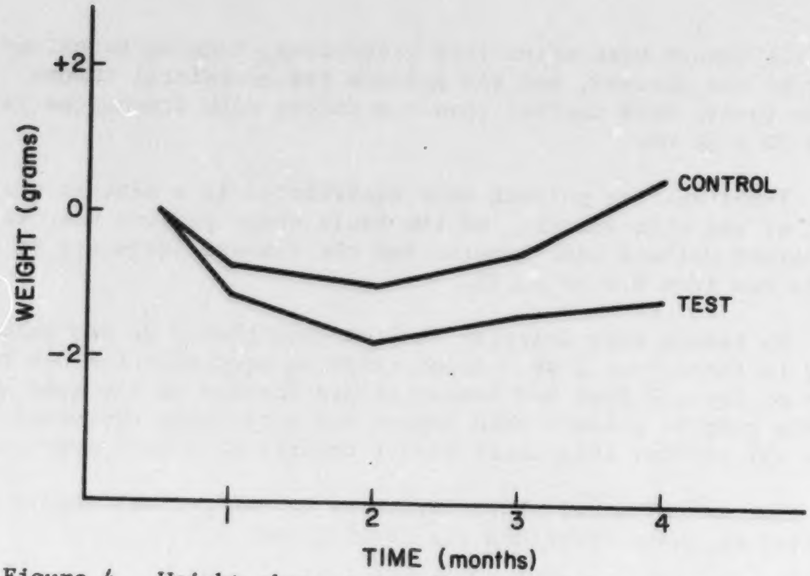

Figure 4. Weight changes in oil-exposed (test) and control English sole over a period of 4 months. Each point represents the average body weight of 16 and 15 fish for the test and control groups, respectively.

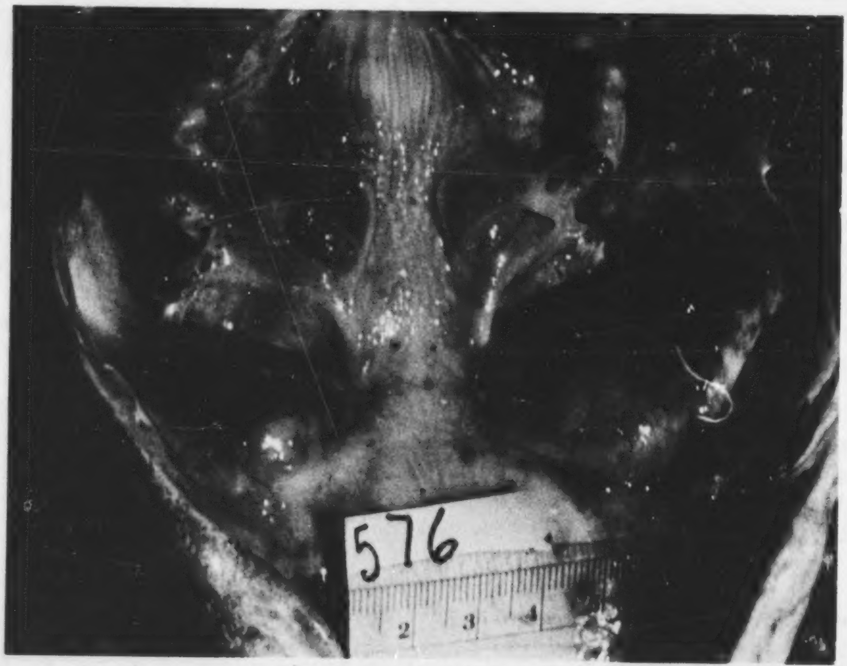

Figure 5. Pacific cod with bilateral pseudobranchial tumors in the pharynx.

pollock tumors were often less protruding, tending to extend up into the roof of the pharynx, and six pollock had unilateral tumors. In general, these tumors were smaller than cod tumors with dimensions ranging up to 35 x 20 x 10 mm.

Tumor-bearing pollock were distributed in a pattern very similar to that of cod with tumors. Of the hauls where pollock were captured, 45% contained pollock with tumors, and the disease frequency in these latter hauls was from 0.6 to 13.2%.

No tumors were detected in fish less than 2 yr and only occasionally in those over 5 yr. About twice as many male pollock between the ages of two and five had tumors as did females of the same age. The growth rate of pollock with tumors was apparently depressed in that they were 15% shorter than their normal cohorts at 3 to 5 years of age.

The histological characteristics of the pollock tumors were very similar to those described for Pacific cod.

The epidermal papillomas of rock sole grossly and histologically resembled similar tumors described for several speices of pleuronectids along the western coast of North America (McCain et al. 1977b; Brooks et al. 1969; Wellings et al. 1967). The tumors ranged in size from 3 x 3 x 2 mm to 100 x 70 x 10 mm. The were brown to black and elevated, with a papillary architecture (Fig. 7). The tumors were located randomly on the body surface, and frequently a tumor extended to both sides of a fish with both sides being mirror images of each other. No metastases were identified.

There were approximately three times as many male rock sole with tumors as females with tumors. The frequencies of tumorbearing fish ranged from 0.4 to 58%, but fish with tumors were found in only 14% of the hauls containing rock sole. The distribution of tumor-bearing rock sole appeared to be depth related, with the shallowest stations (47 to 55 meters) having the highest frequencies. The length frequency distributions of the rock sole examined and for those with tumors demonstrates that this condition is most common among fish, especially males, between the lengths of 8 and 20 cm (corresponding to 2 to 4 years of age).

Lymphocystis (a virus-caused disease) of yellowfin sole was characterized by the presence of variously shaped and colored growths on fins and body surfaces (Fig. 8). These growths ranged in size from 1 mm in diameter to 20 x 10 x 5 mm. Most growths were found on the "blind" side of the fish.

Diseased yellowfin sole were most often observed in the southeastern Bering Sea, just north of Unimak Island. Of the hauls containing this species, 34% had fish with lymphocystis with frequencies ranging from 2.3 to 31.3%. About the same proportion of males as females had this disease. The length frequency distributions of the diseased and the total fish examined were very similar.

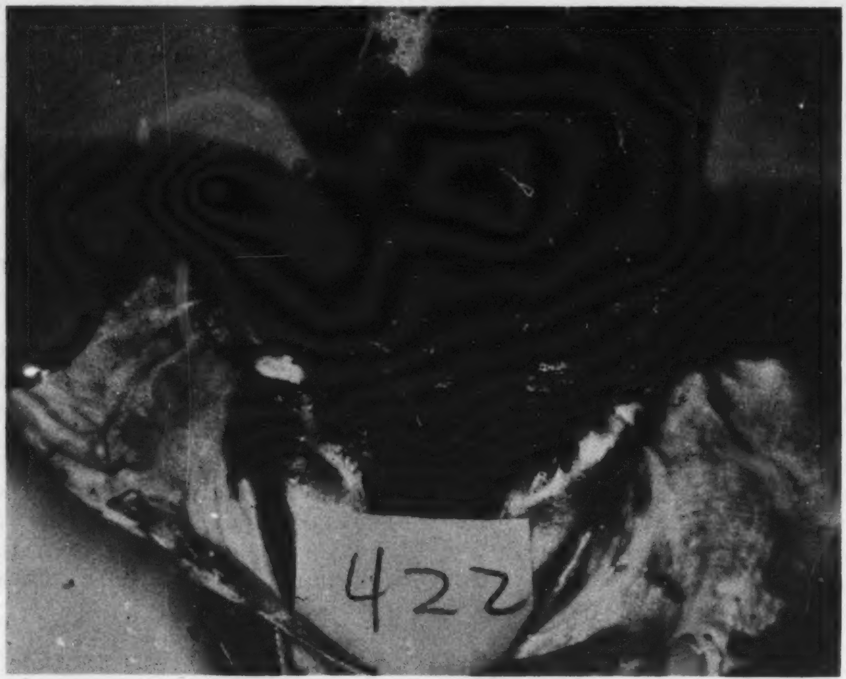

Figure 6. Pollock with bilateral pseudobranchial tumors (just above card) in the pharynx.

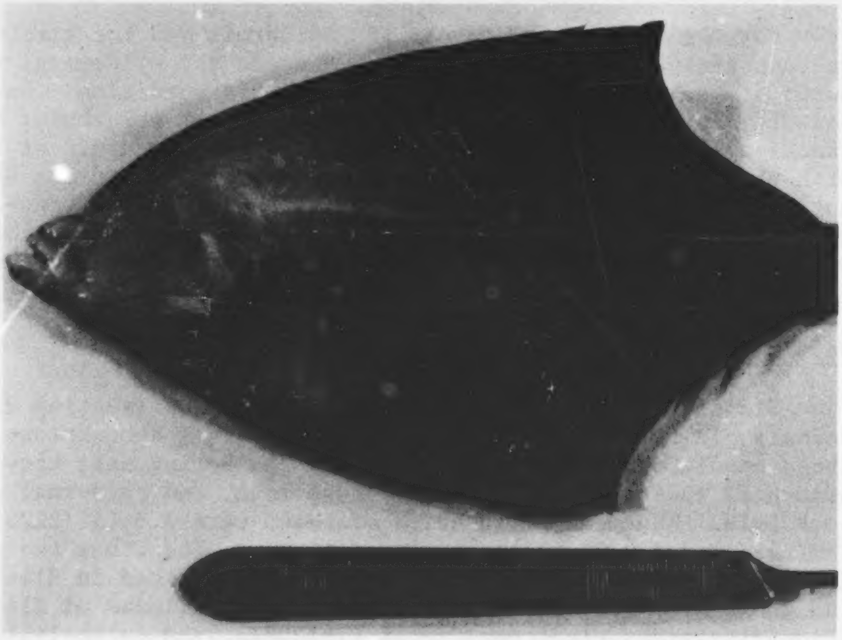

Figure 7. Rock sole with a skin papilloma on the blind side body and anal fin.

The histological properties of lymphocystis growths have been extensively described elsewhere (McCain et al. 1977b; Russel, 1974; Templeman, 1965). The growths on yellowfin sole contained hypertrophied cells about 0.1 to 1.5 mm in diameter. These cells contained cytoplasmic inclusion bodies which were composed to hexagonal virions about 200 nm in diameter.

Two main types of skin lesions were observed on Pacific cod: an ulcer (Fig. 9) and a ring-shaped lesion (Fig. 10). The ulcers were roughly circular and ranged in size from approximately 1 to 50 mm in diameter. The ulcers were either pale white, or red (hemorrhagic) with a dark pigment concentrated in the margin of the surrounding epidermis. Between one and 25 ulcers were observed on each affected fish. The ring-shaped lesions were characterized by a 5 to 20 mm wide, cream-colored strip, sometimes having hemorrhagic foci, surrounding a normal-appearing circular patch of epidermis. These lesions were about 10 to 50 mm in diameter. The number of ring-shaped lesions per diseased fish ranged from one to five. The highest frequencies of skin lesions were found on fish caught northwest of Unimak Island. Both sexes of cod had about the same frequency of skin lesions.

The histological properties of the skin ulcers included a loss of epidermis, and a marked host response consisting of inflammation and fibrosis. Attempts to isolate bacteria from these ulcers yielded taxonomically identical pseudomonas-like bacteria from 5 different Pacific cod.

For the ring-like lesions, the epidermis and the stratum spongiosum (a component of the dermis directly beneath the epidermis) were the only parts of the skin obviously affected by this condition. Cod epidermis normally contains mucous cells and large cystic structures of unknown function (Bullock and Roberts, 1974). In the epidermis of fish affected with the lesion there were large bodies, about four times the size of a normal mucous cell, which contained a very basophilic center surrounded by an eosinophilic margin. Electron microscopic examination of these basophilic bodies demonstrated the presence of herpes-like viruses (McArn et al. 1978).

Gulf of Alaska

Six of the 35 species of fish examined, representing 25,633 total animals, in the GOA had four major pathological abberations. Two of these conditions were also observed in the Bering Sea; they are pseudobranchial tumors of Pacific cod and pollock, and epidermal papillomas of rock sole. The mean frequencies of these were 2.3 (52/2225), 0.7 (39/5437), and 0.2% (3/1925), respectively. The other two pathological conditions, which have not previously been reported in Alaskan waters, and their mean frequencies were epidermal papillomas of flathead sole (*Hippoglossoides elassodon*), 0.4% (10/2521), and epithelial tumors of Pacific Ocean perch (*Sebastes alutus*), 0.7% (16/2466).

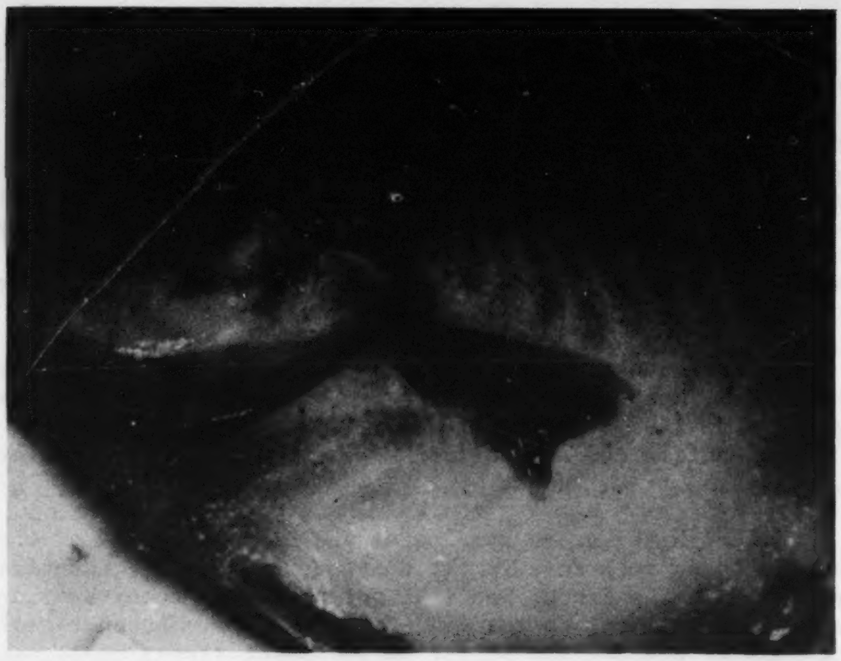

Figure 8. Yellowfin sole with a lymphocystis growth on the blind side pectoral fin. Note the loss of fin tissue.

Figure 9. Pacific cod with several skin ulcers on the body surface.

The gross appearance and histopathology of the pseudobranchial tumors of pacific cod and pollock from the GOA were very similar to what has been described for this disease in the Bering Sea. In addition, about the same number of male cod had tumors as did females in both areas. The age and sex composition of tumor-bearing pollock in the two areas differed slightly. Pollock with tumors between the ages of two and four from the Bering Sea had the highest tumor frequency, while all the tumor-bearing pollock from the GOA were two years old. Also, twice as many male pollock in the Bering Sea had tumors as did females, but in the GOA, three times more males than females had tumors.

The frequencies of tumor-bearing Pacific cod and pollock in hauls in the GOA where pseudobranchial tumors were observed in these species ranged from 1.0 to 50% for cod and 0.32 to 14% for pollock. Tumor-bearing Pacific cod and pollock were evenly distributed over the sampling area.

Although the gross appearance and histopathology of the epidermal papillomas on rock sole in the GOA were essentially identical to what was found in the Bering Sea, the prevalence of tumor-bearing rock sole was very much lower in the GOA (0.2 vs. 1.1%). Another similarity between the GOA and the Bering Sea, was the strong relationship between shallowness of the sampling station and a high frequency of tumor-bearing rock sole (Mark Myers, unpublished data).

The epidermal papillomas of flathead sole were very similar in appearance to the rock sole skin tumors. The ages of tumor-bearing flathead sole ranged from 4 to 11 years, with an average age of 6.5 years. The distribution of tumor-bearing flathead sole followed an inshore pattern similar in nature to that reported for tumor-bearing rock sole in the Bering Sea.

The epitheliod tumors in Pacific Ocean perch were located primarily on the underside of each operculum (Fig. 11) but in some cases they had spread onto the gill arches and body surface. No gross tumors were observed in internal organs. The ages of the affected fish ranged from 9 to 16 years (Myers et al., unpublished data).

Examination of histological preparations of the perch tumors revealed that they have a cellular structure very similar to the pseudobranchial tumors of cod and pollock and that they contain tumor-specific cells closely resembling the X-cells found in the cod and pollock tumors as well as the epidermal papillomas of flatfish.

Norton Sound/Chukchi Sea

A single pathological condition was found in 3 of the 28 species of fish examined, representing a total of 26,901 fish. This condition is called "black spot" disease and is characterized by the presence of

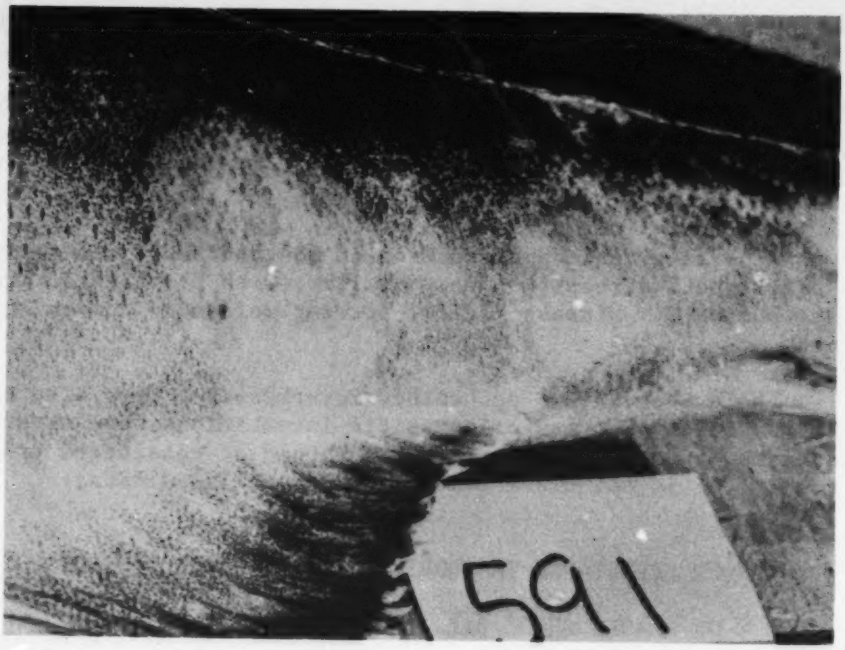

Figure 10. Pacific cod with two ring-shaped lesions near the caudal region.

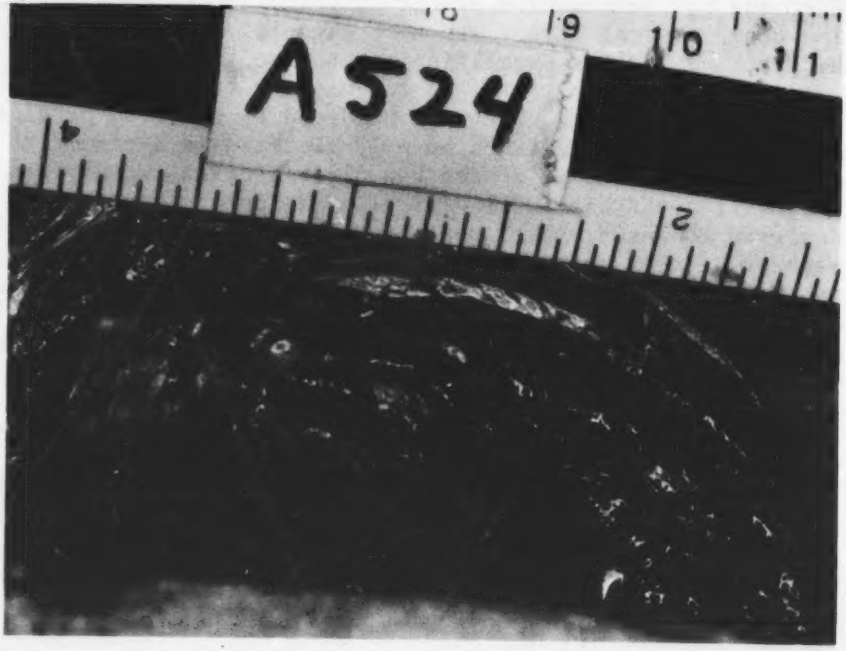

Figure 11. Pacific Ocean perch with several epidermal tumors on the underside of an operculum.

black cysts (Fig. 12) containing a larval trematode (probably *Cryptocotyle lingua*) surrounded by melanin-containing cells. The species involved and the overall frequency of this condition were: Pacific herring (*Clupea harengus pallasi*), 4.5% (92/2027); the toothed smelt (*Osmerus mordax dentex*), 3.9% (146/3772); and the saffron cod (*Eleginus gracilis*), 0.8% (91/10,826) (Bill Gronlund and Kate King, unpublished data).

The geographic distribution of toothed smelt and saffron code with "black spot" disease was very similar, with the highest prevalence in Norton Sound. Disease Pacific herring were more common in the Chukchi Sea.

More than 25,000 epibenthic invertebrates were also examined in this area and 5 types of parasitic infestations were observed in 5 species, but whether or not these should be considered true pathological conditions is not clear (Kate King, unpublished data).

RELEVANCE TO PROBLEMS OF PETROLEUM DEVELOPMENT

EFFECTS OF ALASKA CRUDE OIL

English sole undergoing long-term exposure to Alaskan crude oil-contaminated sediments developed two general types of pathological conditions. One type was an acute hepatic response resulting in severe HLV, and was accompanied by the presence of certain PAHs in the liver. The other type of effect was more chronic, leading to emaciation, morbidity, and greater parasitic infestation.

Several factors must be considered in order to predict if crude oil would have similar effects on wild flatfish as a result of a major oil spill. At present, it is not known if flatfish, when given the opportunity, will avoid harmful levels of petroleum hydrocarbons. Experiments designed to answer this question will be performed this year by other members of this research unit. The energy level of the beaches involved in the spill (i.e., ranging from high energy beaches with active surf to low energy beaches within sheltered bays) will affect the levels of hydrocarbons in the sediment and the length of time they persist. The species of flatfish near the spill may not all develop the same pathological conditions in response to oil. Also, the degree of weathering of oil may be an important factor in its ability to induce pathology.

Nevertheless, the best available knowledge suggests that English sole, and probably most other Pleuronectidae, exposed to levels of petroleum hydrocarbons of 500 ppm or higher in the sediments of a low energy beach for several months will develop pathological anomalies similar to those observed in the above-mentioned laboratory experiments.

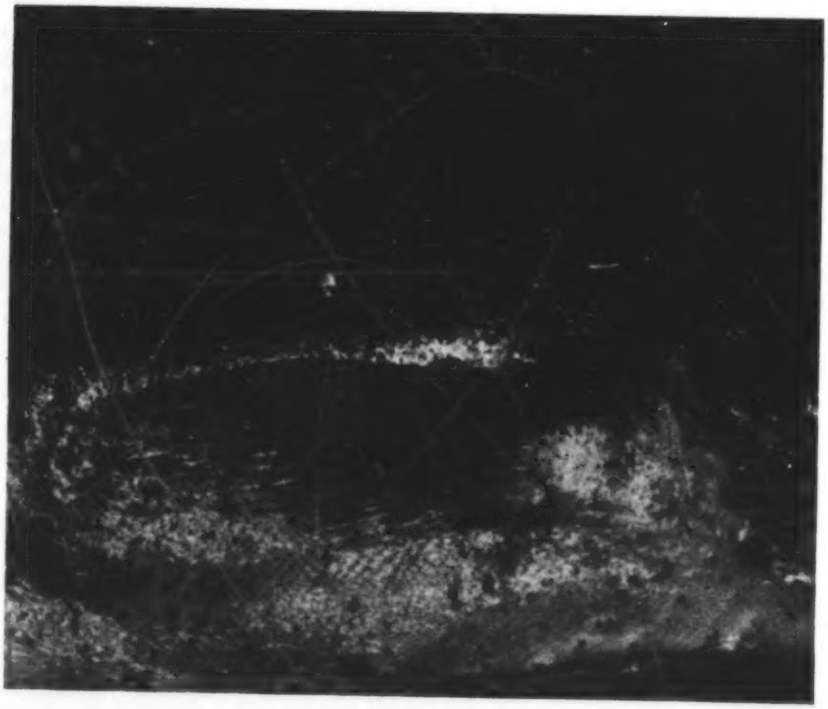

Figure 12. Saffron cod with numerous black trematode cysts in the skin.

BASELINE FISH DISEASE STUDIES

The principal contribution of these baseline studies has been the demonstration that significant frequencies of disease are presently in the demersal fish populations associated with Alaska's Outer Continental Shelf. In addition, these studies have provided information concerning the species free of detectable disease and those species affected by specific diseases, the geographic distribution of affected fish, and the possible causes of some of the diseases. Therefore, in the aftermath of possible incidents of crude oil contamination of Alaskan marine waters, the simple existence of diseased fish will not be sufficient to show the harmful effects of oil. If, however, certain aspects of pathological conditions deviate significantly from the parameters established during the above-mentioned baseline studies, then oil contamination could be implicated as a possible cause. Examples of deviations from the baseline parameters could be: (1) The finding of diseased fish of a species previously found to be disease-free, (2) a major increase in the frequency of certain diseases in particular species, and (3) the finding of a disease in fish in a geographical area where it had not been previously detected.

PROBLEMS ENCOUNTERED

No significant problems were encountered with either project.

RECOMMENDATIONS FOR FUTURE RESEARCH

EFFECTS OF ALASKAN CRUDE OIL

As part of a continuing effort to better understand the pathological effects of crude oil on flatfish, we plan during the current year, to compare response to long-term exposure to crude oil-contaminated sediments of at least three different species of flatfish common to Alaska. In the future, we would also like to determine the long-term effects of exposing the pelagic life stages (eggs and larvae) of flatfish to seawater soluble (SWS) fractions of crude oil. Several species of flatfish along the west coast of North America, including Alaska, have skin papillomas at epizootic incidences (Hodgins et al. 1977). Because these skin tumors develop on flatfish during their first year, it is likely that tumor-induction may occur during or shortly after the larval stage. In addition, starry flounder (Platichtys stellatus), a flatfish species which has a high prevalence of skin tumors, when exposed to SWS fractions of crude oil, bioconcentrate aromatic hydrocarbons in tissues by factors as high as 11,000 (Roubal et al. 1977b). When one such hydrocarbon, naphthalene, was fed to starry flounder, it was actively metabolized, and accumulations of metabolites were found to persist in the liver, blood, and skin for over one week (Varanasi and Gmur, 1978). Metabolites of the carcinogenic forms of PAH are known to be the most carcinogenic form (Varanasi and Malins, 1977). The proposed experiment procedure would involve exposing the eggs and larvae to SWS crude oil, raising the larvae through maturity until they are at least 6 mo old, and monitoring them for pathological changes. Such pathological changes may include liver abnormalities, microbial or parasitic diseases, fin erosion, or skin lesions.

Another aspect of our work with flatfish and the effects of oil exposure that could be considered for future research would be the examination of flatfish, and possibly other demersal fish species, associated with actual oil spills. By examining such fish using histopathological techniques and petroleum hydrocarbon analyses, a comparison between our laboratory observations and the effects of oil spills on wild fish can be made. Also, sediment samples could be collected near the oil spills, taken to our laboratory, and tested for their long-term pathological effects on selected flatfish species.

The long-term pathological effects of oil-dispersant mixtures on flatfish is also an excellent subject for future research. Such mixtures could be adsorbed to sediment or continually added to a flow-through aquarium system. The protocol for this investigation would be very similar to the procedures now being used in experiments with oil-contaminated sediments.

BASELINE FISH DISEASE STUDIES

Baseline studies on the health status of Alaskan demersal fish, such as those reported here, are very complementary to resource assessment studies. Fish captured and examined for population studies can also be examined for pathological conditions with only a small increase in time and personnel. Therefore, as long as OCSEAP-supported resource assessment programs are carried out in Alaska, we feel that it would be in OCSEAP's best interest to continue fish disease studies.

Such studies would provide several benefits. By being able to monitor limited geographic areas for disease frequency, distribution, and pathology over a multi-year peiod, the natural fluctuation of certain disease could be estimated, certain "hot spots" of disease occurrence could be identified, and, in the event of extensive oil contamination, possible increases in disease frequency and species affected could be documented.

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PHYSIOLOGICAL AND BEHAVIORAL EFFECTS

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OBJECTIVES AND RATIONALE

Specific objectives of the physiological and behavioral studies were to define and evaluate: (1) effects of water-soluble fractions of crude oil and a model hydrocarbon mixutre on Pacific salmon (*Oncorhynchus*) homing; (2) effects of a water-soluble fraction of crude oil on feeding behavior of spot shrimp (*Pandalus platyceros*); (3) effects of a water-soluble fraction of crude oil on the reproductive behavior, embryogenesis, and larval development of an intertidal invertebrate, the dorid nudibranch *Onchidoris bilamellata*; (4) effects of long-term ingestion of crude-oil-coated food on reproduction of rainbow trout (*Salmo gairdneri*); and (5) effects of exposure to oil in diet or in water on disease resistance of salmon and trout.

These studies were designed as laboratory experiments--with the notable exception of field studies of salmon homing--to evaluate effects of sublethal petroleum exposure on species indigenous to the North Pacific Ocean tested under natural water quality and temperature conditions.

METHODS

EFFECTS OF PETROLEUM ON SALMON HOMING

Studies were initiated in October 1976 to evaluate the effect of petroleum hydrocarbons on the migratory behavior of adult coho (*O. kisutch*) and chinook (*O. tshawytscha*) salmon (Douglas D. Weber and Desmond Maynard, unpublished). Detailed descriptions of methods used are presented in the OSCEAP Annual Report for Research Unit #73/74, April 1977, and will be only briefly overviewed here. Two of the studies, one in the marine environment and the other in freshwater, were designed to determine if short-term exposure to petroleum hydrocarbons had a subsequent effect on salmon homing performance. The third experiment was designed to determine if salmon avoided their home stream when petroleum hydrocarbons were present in the water.

The two studies on the effect of petroleum exposure on homing performance were similar in experimental design, consisting of capture-exposure-transport-release; but differed in species used, petroleum components, and geographical locations. One experiment was conducted at the University of Washington using adult chinook salmon returning to a freshwater pond. After exposure to the water-soluble fraction of Prudhoe Bay crude oil and tagging, these fish were transported 7.8 km downstream from the pond and released; numbers of test (petroleum-exposed) and control (all conditions the same except no petroleum exposure) fish returning to the pond were counted. The other similar experiment was conducted using coho salmon captured at the mouth of their home stream (Beaver Creek) near the NWAFC acquaculture station at Clam Bay on the west side of Puget Sound. Tagged test and control fish were transported 2.1 km offshore in Puget Sound and released, and numbers of each treatment group returning to Beaver Creek or captured as strays in other areas were counted. Fish at the University of Washington were exposed for 14-18 hr to concentrations of Prudhoe Bay crude oil determined by gas chromatographic analysis to range up to 178 ppb. Fish at Beaver Creek (Clam Bay) were exposed for 24 to 45 hr to a model mixture of hydrocarbons (composition given in OCSEAP Annual Report, April 1977) at concentrations up to 256 ppb.

In the third study (effects of hydrocarbons in home stream water on salmon entering) relative numbers of chinook salmon returning to a raceway at NWAFC were compared in relation to the presence or absence of hydrocarbons (the model mixture described above) introduced into the home stream water. Water-soluble hydrocarbon concentrations ranged up to 193 ppb under test conditions, as determined by gas chromatography.

Research with similar or identical experimental designs, but with fish homing to different streams, is in progress to substantiate and extend the results obtained in 1976.

EFFECTS OF PETROLEUM ON FEEDING BEHAVIOR OF SHRIMP

Observations on the feeding response of the spot shrimp were made during six-day exposures to the seawater-soluble fraction of Prudhoe Bay crude oil (10-600 ppb) (Stephen Miller and Douglas D. Weber, unpublished; Stephen Miller, MS Thesis, University of Washington, in preparation). The experiments were conducted at NWAFC's station at Mukilteo utilizing a continuous oil solubilizer system described by Roubal et al. (1977a). Details of methods used are presented in the OCSEAP Quarterly Report for Research Unit #73/74, April 1977.

EFFECTS OF PETROLEUM EXPOSURE ON REPRODUCTION OF A DORID NUDIBRANCH

Adult dorid nudibranchs were exposed to the seawater-soluble fraction of Prudhoe Bay crude oil (13-420 ppb) for 1 or 3 days in flow-through aquaria. Following exposure either to seawater containing oil (test animals) or to seawater without oil (control animals), the nudibranchs were observed for responses consisting of joining or not joining mating aggregates (Frederick Johnson and Douglas D. Weber, unpublished). The joining of mating aggreagates is a chemotactic behavioral response which is thought to be pheromone-mediated and necessary for successful

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mating. For this study a response was defined as movement out of the initial chamber in a choice apparatus (Fig. 1). The stimulus chamber contained an aggregate of four control nudibranchs and the blank chamber was without nudibranchs. Further details of methods used in this study are in the OCSEAP Quarterly Report for Research Unit #73/74, June 1977.

In another experiment, twenty mature dorid nudibranchs were placed in each of four flow-through aquaria supplied with filtered seawater containing the following concentrations of the seawater-soluble fraction of Prudhoe Bay crude oil: 0, 8, 27, or 278 ppb. Egg masses were laid by the nudibranchs and were placed in rearing cages in the aquaria. Replicate egg aliquots from these masses were taken daily 5 days per week in buffered preservative (Hawkes, 1974, with addition of 17 g "Instant Ocean" per liter of buffer). In addition, eggs laid each day were collected and weighed, and a count was made of nudibranchs present in aggregations. Analysis of ontogenetic development was conducted using light microscopy (Laura Mumaw and Douglas D. Weber, unpublished; Laura Mumaw, MS thesis, University of Washington, in preparation). These studies are discussed in the OCSEAP Quarterly Report for Research Unit #73/74, September 1977.

EFFECTS OF CRUDE OIL ON TROUT REPRODUCTION

Three-year-old rainbow trout which were maturing for the first time were fed either food soaked in Prudhoe Bay crude oil (1,000 ppm) dissolved in a volatile solvent (trichlorotrifluoroethane), which was subsequently evaporated, or food treated with solvent only. The fish were fed one or the other of the diets for six to seven months prior to spawning, and reproductive successes of the test and control groups were compared. Details of methods used are presented in the OCSEAP Annual Report for Research Unit #73/74, April 1977 and in Hodgins et al. (1977).

EFFECTS OF PETROLEUM ON DISEASE RESISTANCE OF SALMONIDS

A series of *in vivo* and *in vitro* tests were performed to assess the effect of petroleum hydrocarbons on salmonid fish defense mechanisms against infectious diseases (Michael Schiewe, Harold Hodgins, Howard Etlinger, and Jacques Chiller, unpublished).

In one experimental approach, juvenile coho salmon that had received Prudhoe Bay crude oil (1,000 ppm) added to fish food were challenged by injections of the pathogenic bacterium *Vibrio anguillarum*, and resultant mortalities compared with mortalities of similarly-treated, non-oil-fed controls. This study was done using fish in freshwater.

In a second experiment, disease resistance of juvenile coho salmon exposed for 14 days to the seawater-soluble fraction of Prudhoe Bay crude oil (0.8 ppm) in a flow-through saltwater system (Roubal et al. 1977a) was compared to disease resistance of similar non-oil-exposed controls. Bacterial challenge was achieved by injecting different numbers of pathogenic V. anguillarum bacteria into fish in saltwater at 10°C.

In another experiment, the ability to form antibodies was compared among groups of juvenile rainbow trout which were maintained either on standard pelleted fish food or on the same food to which Prudhoe Bay crude oil had been added to form high (1,000 ppm) or low (10 ppm) oil-in-food concentrations. Antibody production was evaluated by determining titers of serum agglutinins for V. anguillarum in test and control fish and by quantitating antibody-producing cells in spleens and anterior kidneys using a Jerne plaque procedure modified for use with fish (Chiller et al. 1969). These studies were described in the OCSEAP Quarterly Report for Research Unit #73/74, June 1977).

In a fourth series of studies, juvenile rainbow trout were given food containing either high or low levels (as above) of crude oil or no added crude oil for 11 months prior to testing and then exposed to V. anguillarum antigens (i.e., the fish were immunized). These fish were then challenged with injection of pathogenic V. anguillarum to directly assess the effect of oil in the diet on their ability to develop adaptive antibacterial immunity. This series of studies is discussed in the OCSEAP Quarterly Report for Research Unit #73/74, September 1977.

RESULTS

EFFECTS OF PETROLEUM ON SALMON HOMING

Exposure of homing adult salmon to petroleum hydrocarbons did not detectably affect their homing capability in the 1976 experiments. There was no significant difference (P=0.25) between the return of captured-exposed-transported-released control and oil-exposed salmon to either the flume at the University of Washington or to Beaver Creek, their respective home streams.

Similarly, the introduction of petroleum hydrocarbons at levels up to 193 ppb directly into home stream water did not prevent adult salmon from entering their home stream in the 1976 studies (Fig. 2). There are strong indications, however, that higher levels of hydrocarbons did inhibit salmon migration in the 1977 studies. These studies will be discussed in a subsequent OCSEAP report after they are completed.

EFFECTS OF PETROLEUM ON FEEDING BEHAVIOR OF SHRIMP

Exposure to the seawater-soluble fraction of Prudhoe Bay crude oil caused a decrease in feeding activity of spot shrimp particularly in those activities involving searching and contact with the stimulus source. The most striking results are illustrated in Figure 3.

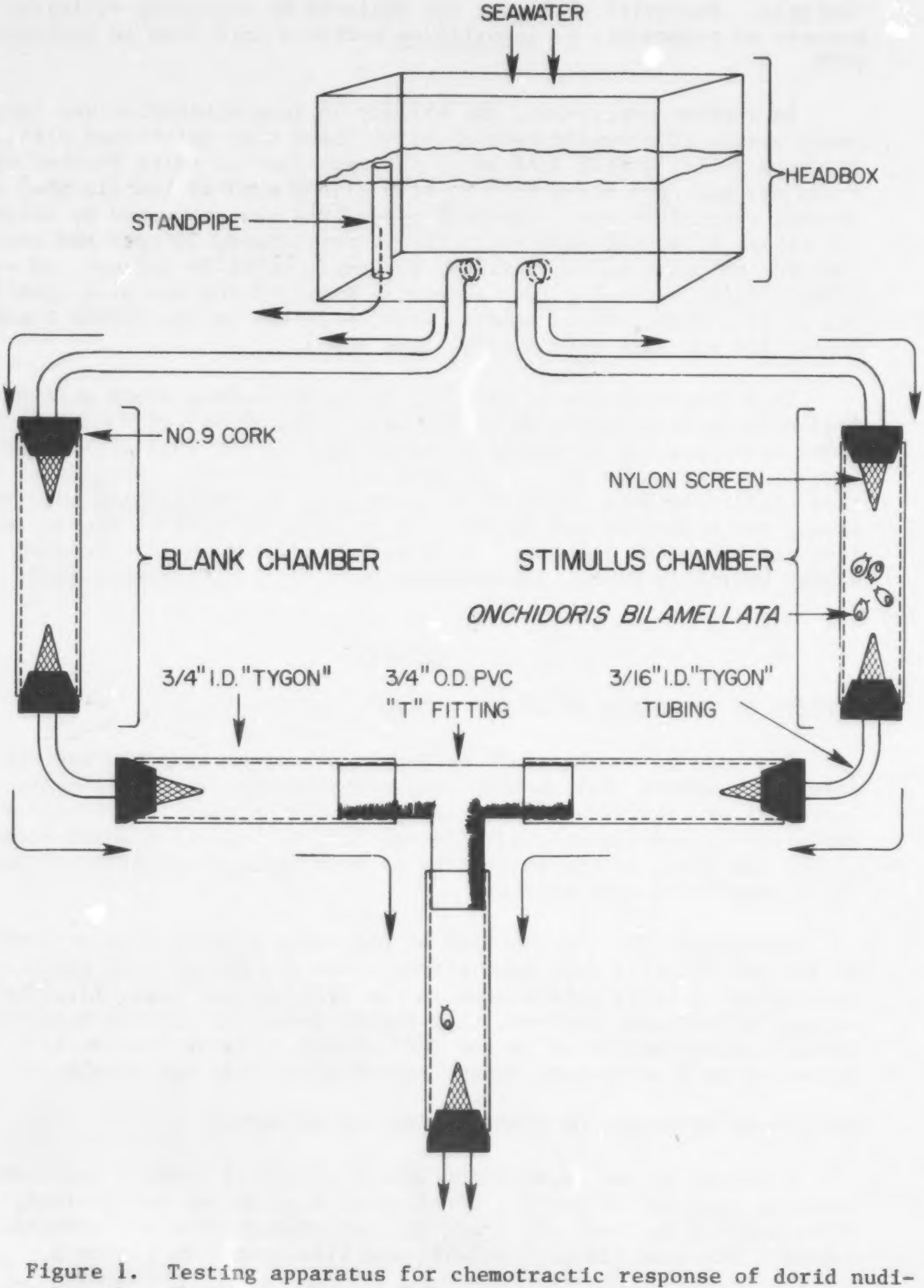

Figure 1. Testing apparatus for chemotractic response of dorid nudibranch. Choice chamber is "T" fitting in lower center of diagram.

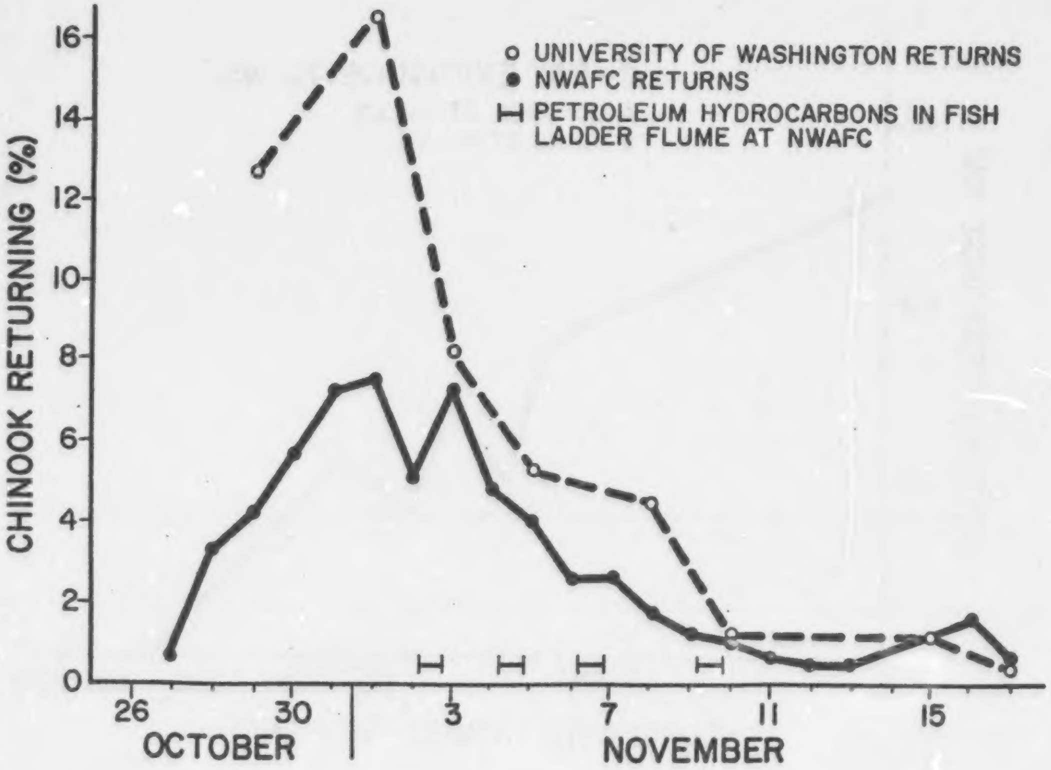

Figure 2. Chinook salmon returning to "home stream" flumes at the University of Washington (UW) and the Northwest and Alaska Fisheries Center (NWAFC) in 1976. Petroleum hydrocarbons were introduced into the NWFC flume for 16-hour periods as depicted by horizontal lines above. No hydrocarbons were added to the UW flume. The UW data is shown as a control because the runs to the two flumes normally have similar timing. A total of 1775 fish returning to the UW are represented by percentages shown and 349 fish returning to NWAFC.

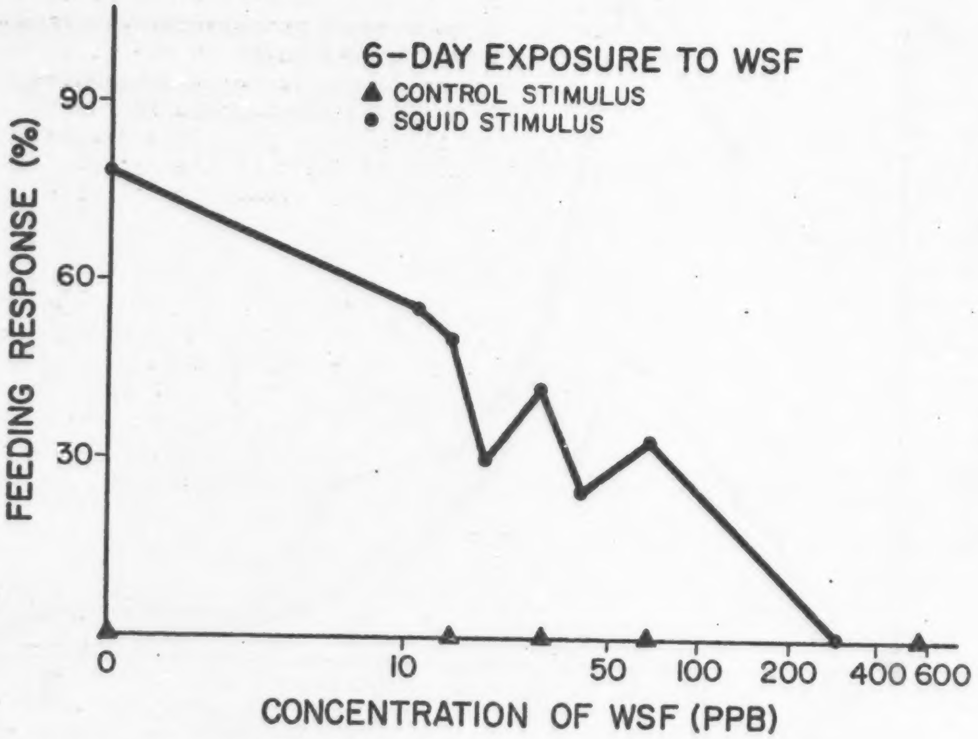

Figure 3. Feeding response (%) of spot shrimp. Each data point is the average of three, 3-minute observations of background activity, response to seawater control, and/or response to a 1:10 dilution of squid extract. A total of 6 control and 27 oil-exposed shrimp were tested. WSF refers to the water-soluble fraction of Prudhoe Bay crude oil.

EFFECTS OF PETROLEUM EXPOSURE ON REPRODUCTION OF A DORID NUDIBRANCH

There was a marked difference between control nudibranchs and those exposed to oil in their movement toward the chamber containing aggregated nudibranchs (Table 1). Whereas almost all (16 of 19) non-petroleum-exposed (control) nudibranchs moved toward the aggregated nudibranchs (see Fig. 1), the movement of petroleum-exposed nudibranchs was random, that is about 50% of the animals that responded moved in the direction of the blank chamber and 50% moved in the direction of the stimulus chamber. In each instance, only about 10% of the animals failed to respond. The chemotactic behavior of the one-day petroleum-exposed nudibranchs was significantly different ($P=0.05$) from that of the controls and the data after three days of exposure showed an even greater difference.

In the ontogenetic studies, developmental delay was evident at the highest hydrocarbon exposure concentration (278 ppb) with some eggs never advancing beyond the single-celled stage. Also, at the highest hydrocarbon concentration, the incidence of abnormalities was nearly 50%, which was much higher than that of controls (0.2 to 4.6%). One hundred percent of the severe abnormalities of the group exposed to 278 ppb involved nonencapsulation. It is virtually certain that this abnormal development results in lack of hatching or larval survival.

EFFECTS OF CRUDE OIL ON TROUT REPRODUCTION

No effect on timing of maturation was evident as a result of petroleum exposure. No statistically significant difference was observed in hatching success (percent survival) of fertilized eggs from crosses in which sperm was used from petroleum-fed or non-petroleum-fed males (Table 2); nor was there a significant difference in hatching success from crosses made to compare eggs from petroleum-exposed or non-petroleum-exposed females (Table 3). Survival of alevins (through yolk sack absorption stage) was higher for control offspring (91%) than for offspring from petroleum-exposed parents (76%), but again the difference was not statistically significant ($P=0.10$).

EFFECTS OF PETROLEUM ON DISEASE RESISTANCE OF SALMONIDS

Neither exposure of coho salmon in fresh water to crude oil in diet nor exposure of cohos to the seawater-soluble fraction of crude oil drastically altered their resistance to bacterial disease under the conditions of the experiments performed. The LD_{50} of _V. anguillarum_ for fish on the diet of crude oil was $10^{-6.0}$, whereas the LD_{50} for control fish on the same diet but without added oil was nearly identical, $10^{-6.6}$. Similarly, there was no significant difference (at the 5% level of probability) in mortalities between bacteria-challenged cohos from petroleum-containing seawater and bacteria-challenged cohos from seawater without added petroleum.

Table 1. *Numbers of nudibranchs (Onchidoris bilamellata) responding (movi into one or the other arm of the "T") in the apparatus shown in FIG. 1. SWSF-exposed animals had been held for 1 or 3 days in seawater containing the seawater-soluble fraction of Prudhoe Bay crude oil at either 13, 61, or 420 ppb and controls were held in seawater without added petroleum. Tests were run in clean seawater and a positive response means the animal moved toward the breeding aggregate. A negative response mwans the animal moved into the (blank) arm that held no nudibranchs.*

	RESPONSE		
	No. Positive	No. Negative	No. No Movement
Control	16	1	2
SWSF-EXPOSED	22	21	5

Table 2. *Comparison of hatching success of fertilized eggs from rainbow trout matings in which the sperm was either from males (test) fed Prudhoe Bay crude oil for 6-7 months prior to spawning or from males (control) fed the same food without added petroleum.*

Sperm Source	No. of Crosses	Mean % Survival Through Hatching
Control Male	16	82.0
Test Male	15	86.4

Table 3. *Comparison of hatching success of fertilized eggs from rainbow trout matings in which the eggs were either from females (test) fed Prudhoe Bay crude oil for 6-7 months prior to spawning or from females (control) fed the same food without added petroleum.*

Egg Source	No. of Crosses	Mean % Survival Through Hatching
Control Female	5	90.3
Test Female	21	86.4

Table 4. *Agglutinating antibody formation in immunized rainbow trout fed Prudhoe Bay crude oil (PBCO)-coated food and immunized or non-immunized trout receiving food without added oil. Values are geometric mean titers (*$\log_2$*) of 10 fish from each exposure regimen.*

Level of PBCO Added in Diet	Mean Antibody Titer Against *V. anguillarum* Bacterin
High (1,000 ppm)	4.3
Low (10 ppm)	5.2
None, Immunized	5.7
None, Non-immunized	<1

The levels of bacteria-agglutinating antibody activity in serum of rainbow trout to crude oil in the diet were little different from those of non-oil-exposed controls (Table 4). Also, in a small number of tests, the number of antibody-forming cells/10^6 spleen cells and the number of antibody-forming cells/10^6 anterior kidney cells did not substantially differ between oil-fed and control trout. There was also no difference in percent mortality between immunized, bacteria challenged rainbow trout which had been fed either high or low doses of petroleum for 11 mo prior to challenge and trout on the same diet without added petroleum (Fig. 4).

There was, however, a significant difference ($P=0.05$) in spleen weights between oil-fed and control rainbow and a pronounced reduction in total splenic leukocytes (Table 5) in oil-fed fish versus controls. The mean ratio of spleen weight (mg) to body weight (g) (x 100) was 69 for controls and 49 for the high-oil-fed goup. Studies are in progress to determine if this reflects a generalized depletion of lymphoid tissue as a result of oik exposure or if the effect is limited to the spleen.

Table 5. Total number of splenic leukocytes in rainbow trout exposed to Prudhoe Bay crude oil (PBCO) in diet for 11 months and in non-oil-exposed control trout. Each value represents pooled cells from spleens of five fish

	PBCO-Concentration Exposure Group		
	1,000 ppm (High oil)	10 ppm (Low oil)	None (Control)
No. of splenic leukocytes	13×10^6	35×10^6	60×10^6

RELEVANCE TO PROBLEMS OF PETROLEUM DEVELOPMENT

These studies have provided clear implications that in certain instances heavy exposures of fish to petroleum have little effect on physiology; whereas, in other instances, severe effects may result from light to moderate exposures to petroleum. In the category of "severe exposure--little effect" are the studies of oil in the diet on trout reproduction, and the disease resistance research with salmon and trout. In both of these studies high doses of crude oil in diets induced little or no detectable physiological impairment. Conversely, exposure of spot shrimp and dorid nudibranchs to ppb levels of petroleum in seawater resulted in potentially disastrous effects on feeding behavior, reproductive behavior, or embryogenesis. Other studies, such as effects of petroleum on salmon migration, may fall somewhere between the above two extremes.

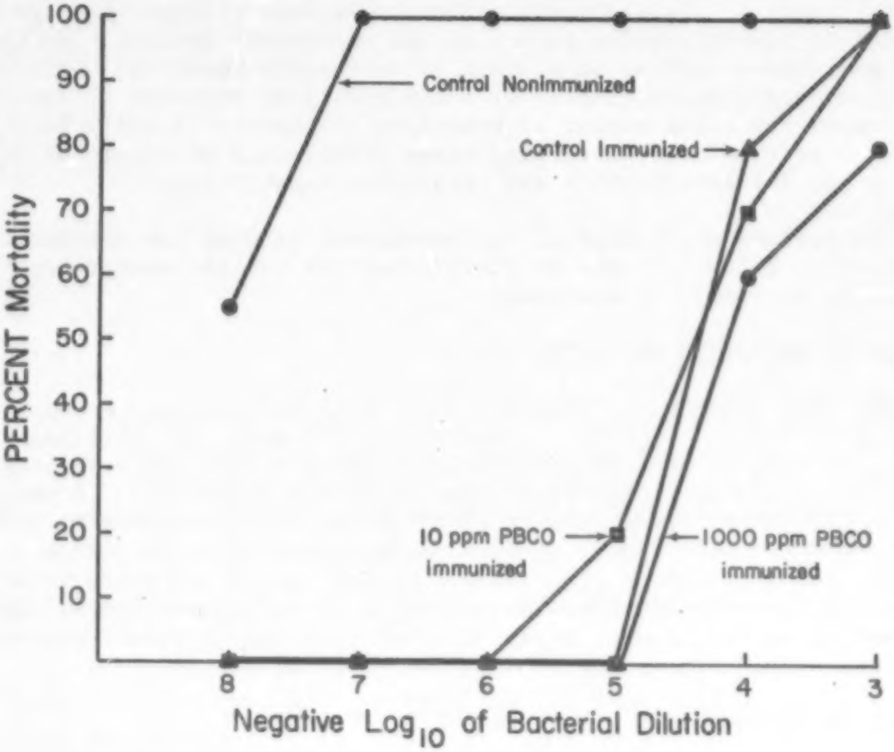

Figure 4. Mortality patterns among Prudhoe Bay crude oil PBCO-exposed and non-oil exposed rainbow trout challenged with V. anguillarum.

It is abundantly clear from our research and the research of others that a host of variables impinge on the effects produced by petroleum on marine species, not the least of which is species variability. The best interpretation we can make concerning the present data is that sublethal exposure to petroleum does under certain circumstances, and does not in others, result in physiological and/or behavioral changes that are inimical to the well-being of marine and anadromous species. Whether or not a particular species in a given natural environment will be significantly damaged physiologically or behaviorally by exposure to any combination of the large number of petroleum components is still largely unknown. As knowledge concerning these effects and interactions increases so will interpretive and predictive capabilities.

The various physiological and behavioral studies are discussed individually below in terms of their potential use in interpreting effects of petroleum development:

EFFECTS OF PETROLEUM ON SALMON HOMING

The 1976 studies revealed no effect from hydrocarbons on homing behavior of salmon. However, research in 1977 indicated that higher doses than used in 1976 of water-soluble hydrocarbons added to home stream water did interfere with salmon upstream migration. If this finding is substantiated, the implication are that petroleum in spawning streams and estuaries could at least delay spawning migrations and perhaps more seriously disrupt them. Whether or not exposure to petroleum in saltwater affects homing salmon is presently not known. Some information on this aspect is anticipated from the capture-exposure to petroleum-transport-release studies currently in progress.

EFFECTS OF PETROLEUM ON FEEDING BEHAVIOR OF SHRIMP

Exposure to ppb of water-soluble petroleum fractions inhibited feeding activity of spot shrimp in the laboratory. The effect seemed to be primarily on the chemosensory system rather than due to narcosis. It is likely that a similar effect would occur under natural conditions.

EFFECTS OF PETROLEUM EXPOSURE ON REPRODUCTION OF A DORID NUDIBRANCH

Clearly, exposure to ppb levels of the seawater-soluble fraction of Prudhoe Bay crude oil in laboratory studies disrupted reproduction of the dorid nudibranch Onchidoris bilamellata. It seems highly probable that similar effects would occur with free-living nudibranchs. It is not presently known if these results should be interpreted as primarily isolated specific effects on one intertidal marine invertebrate's chemoreception and embryogenesis, or if they are much more generalizable findings.

EFFECTS OF CRUDE OIL ON TROUT REPRODUCTION

The implications from these studies are that chronic exposure to large amounts of unweathered Prudhoe Bay crude oil components in the diet of salmonid fishes (at least, Salmo gairdneri) do not significantly interfere with hormonal and other biochemical processes of maturation needed to produce viable gametes and offspring. It should not be inferred from this research, however, that exposure to crude petroleum clearly does not affect salmonid reproduction, because of possible species differences and a number of other parameters such as stamina, redd building, etc., which were not investigated.

EFFECTS OF PETROLEUM ON DISEASE RESISTANCE OF SALMONIDS

These studies revealed no effect from petroleum on the ability of salmonid fishes to form antibodies or resist laboratory-induced bacterial infections. The markedly reduced spleen sizes in crude-oil-exposed trout suggests that a so-far-undetected functional impairment in mechanisms of immunity may exist. If the effect is limited to the spleen, it would presumably be of less consequence than if a general reduction in lymphoid tissue is found.

PROBLEMS ENCOUNTERED

No problems were encountered that prevented completion of the principal elements of the proposed research.

RECOMMENDATIONS FOR FUTURE RESEARCH

Recommended future behavioral and physiological research includes studies of:

1. Avoidance of oil and oil-dispersant mixtures by juvenile salmon and flatfish in laboratory studies.

2. Effects of oil-dispersant mixtures on salmon homing.

3. Effects of petroleum and petroleum-dispersant mixtures on immune responses and disease resistance of flatfish, and effects of petroleum-dispersant mixtures on disease resistance of salmonids.

4. Effects of petroleum and dispersants on an activity such as swimming performance which incorporates integration of a number of physiological, biochemical, and behavioral parameters. This would serve as a screening procedure and as a prelude to more in-depth investigations.

5. Field studies on petroleum avoidance or non-avoidance by mobile species released in or near areas of high oil contamination (oil spills) using sonic tags and capture-convetionally tag-release-recapture techniques.

Some of these studies are outlined in further detail in the section entitled "XVI. Outlook" in the proposal to OCSEAP for FY 1978 (dated June 24, 1977) for Research Unit #73.

MORPHOLOGY

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OBJECTIVES AND RATIONALE

The two major objectives are to identify the effects of petroleum on fish at the cellular level and to establish which tissues show the most obvious and repeatable structural alterations. A series of experimental designs have been followed to assess the effects of Prudhoe Bay crude oil or its water-soluble fraction on both pelagic and demersal species and on adult and young. The findings will be reported by considering each major target organ and describing the alterations observed for each type of experiment and variation. The common theme is the type of cellular response of selected organs or tissues to toxic substances; namely, petroleum hydrocarbons.

METHODS

Several species of flatfish and salmonids were exposed to Prudhoe Bay crude oil in the diet and/or the water-soluble fraction of Prudhoe Bay crude oil in the water (Hawkes, 1977a, 1977b, 1977c; Hodgins et al. 1977a; Hodgins et al. 1977b, McCain et al. 1977). The design of each experiment is detailed in other parts of this report as the fish were shared among several investigators in order to obtain data from several disciplines; specific exposure parameters will be described for each target tissue in the results section.

Techniques for preparing tissues for morphological studies were consistent throughout the study and entailed taking replicate samples from three fish of skin, gill, and intestine for scanning electron microscopy (SEM) and skin, gill, liver, intestine, and kidney for light microscopy (LM) and transmission electron microscopy (TEM). In certain studies lens tissue was sampled for both SEM and TEM. The tissues were excised and fixed in 0.75% glutaraldehyde, 3% formalin, 0.5% acrolein in 0.1 sodium cacdylate buffer with 0.02% $CaCl_2 \cdot H_2O$, 0.02 M s-collidine, and 5.5% sucrose (Hawkes, 1974). The tissues designated for light microscopy (LM) or for transmission electron microscopy were post-fixed in osmium tetroxide in the same buffer, dehydrated in an ethanol series, and embedded in plastic. Sections were cut at 0.5 μm, stained with toluidene blue or a trichrome (MacKay and Mead, 1970) for LM. For TEM, sections were cut with a diamond knife and stained with lead citrate and uranyl acetate and examined with a Philips EM-301. For SEM, samples were dehydrated after the initial fixation, critically point dried, coasted with gold-palladium, and examined with an AMR-1000 scanning electron microscope.

RESULTS

GILL

Coho salmon and starry flounder were exposed to 100 ± 90 ppb of the water-soluble fraction of crude oil (WSF) for five days in a flow-through saltwater system (Roubal et al. 1977a). The gills of exposed fish developed lesions which reflected the loss of the surface cells or the first two to three layers of cells. Immature mucous glands below the surface were exposed when the surface sloughed and their contents, in some instances, were exuded, which could account for some of the excess mucous secretion observed in other studies (Blanton and Robinson, 1973). The area of sloughing varied from filament to filament: 10 to 30 cells were lost in the smaller lesions and, in a few cases, the surface of an entire gill filament lost its outermost layer of cells. In both experimental and control coho salmon a gill ecto-parasite was observed, a monogenetic trematode (Gyrodactylus sp.) which needed to be considered in determining if the cellular damage could be the sole result of the parasite. The opishaptor of Gyrodactylus has hooks for attachment to the gill or skin surface, and the attachment does not appear to particularly damage the fish cells. Heavy infestations, however, can cause a diseased state in fish (Mellen, 1928) and should be accounted for in reporting lesions especially with routine histopathological techniques where serial sections would be needed to ascertain the presence of the parasite. In the present study, the unexposed fish had Gyrodactylus and did not have lesions. In this case, the infection was probably low enough not to adversely affect the host. An area for future research could include a study of the relationship between degree of infection with specific parasites or pathogens and possible synergistic or antagonistic effects of particular contaminants which may, in combination, severely affect survival of fish.

SKIN

English sole were exposed to the water-soluble fraction (WSF) of Prudhoe Bay crude oil (Hawkes, 1977a): 10 ml crude oil in 1 liter of seaweater was stirred for 20 hours, allowed to stand for three hours, and the bottom, clear fraction was removed. The WSF was then mixed with seawater to make a 13% WSF solution. The glass tanks were aerated and maintained at 10°C.

In the skin samples of English sole taken five days after the WSF had been added, many of the mucous glands were completely empty. In a repeat experiment, skin samples were taken from three different body locations during a two-hour to five-day time study. Results were inconclusive because there was a great deal of varability in both the numbers of glands and in the number that had discharged their contents. More extensive studies are projected to understand the normal rate of mucous exudation and to define precisely alterations of that process with increasing concentrations of petroleum.

LIVER

Depletion of energy-storage products and infiltration of hepatic blood vessel by connective tissue were found in the livers of rainbow trout that received Prudhoe Bay crude oil in their diets (Hawkes, 1977a; Hodgins et al. 1977a). In one study, 2 ml of crude oil were dissolved in trichlorotrifluoroethane and adsorbed to 1 kg of Oregon moist pellets (OMP). The solvent was allowed to evaporate, and the OMP was fed to the trout at 2% of body weight for five days per week. Assuming no evaporative loss of petroleum components, each fish received 11 mg of crude oil per day and the average weight of the trout was about 90 g. The controls were treated exactly the same as the experimental trout except that crude oil was omitted from the solvent-treated food. Two fish were sampled from each of our replicates of both experimental and control groups.

After two weeks of feeding with no mortality, there were dramatic differences in the levels of energy reserved in the liver: the hepatocytes of control fish were full of glycogen whereas those of the experimental fish had virtually none. These changes were evident in 0.5 μ sections stained with toluidine blue. The polychrome method (MacKay and Mead, 1970), which stains with the mucopolysaccharide moieties bright red when the cytoplasm is blue, was used on 1.0 μ sections to differentiate glycogen deposits in the cells. The sections for TEM analysis showed the same disparity. Proliferation of the endoplasmic reticulum was evident.

In another study of liver morphology, English sole were exposed to 700-400 ppm of Prudhoe Bay crude oil in sediments for 27 days (See: The effects of Alaskan crude oil on flatfish and the prevalence of fish pathology in Alaskan marine waters). With both light and electron microscopy, vacuolization in the liver was moderate to heavy in petroleum-exposed fish and light to moderate in controls. TEM examination of hepatocytes revealed large vacuolar areas filled with numerous liqid droplets of variable sizes. Immediately adjacent to and surrounding the nuclei of the vacuolated cells were whorls of rough endoplasmic reticulum. There was no evidence of necrosis or cell loss in the liver and no changes in the structure of other organelles.

EYE LENS

The same trout that developed liver abnormalities after exposure to crude oil in the diet also had enlarged eye lenses (Table 1) which were abnormally soft (Hawkes, 1977a; Hodgins et al. 1977a). Relatively mild pressure permanently compressed the lenses of exposed fish into an amorphous mass, whereas the control lenses returned to their normal geometry after the same pressure.

Another laboratory has recently found similar lens size increases in cunner, *Tautogolabrus adspersus*, chronically exposed to Venezuelan crude oil for six months in a flow-through seawater system (Payne et al. 1977).

TABLE 1

Volume of eye lenses from trout fed crude oil for one year

Group	X(mm^3)	SX	N
Control	100.9	19.2	4
Oil-treated	226.10	81.2	6

The lens is composed of ribbon-like filaments which interdigitate and form a sphere. The filaments have simple projections on their broad surfaces which plug into pits on the adjacent fiber in addition to a complex interlocking series of protuberances on their thin side. After treatment with petroleum, the fiber structure changed: the broad surface was wrinkled and the interdigitating projects were not smooth and regular as in untreated fish. The fibers looked shriveled, as if the fixation was hyperosomotic, suggesting that the increase in size might be due to hydration of the lens rather than to increased mass resulting from cell proliferation or cell secretory activity. To test the hydration hypothesis, lenses were removed from normal rainbow trout, measured, and placed in a dilution series of "Dulbecco's" saline and distilled water. Hydration occurred at slightly different rates but in approximately five hours there was an 80% increase in volume which stabilized until the termination of the experiment at 45 hours.

To test the uptake of a specific petroleum hydrocarbon, naphthalene, into the lens and other tissues, rainbow trout, each weighing about 100 g, were fed a gelatin capsule containing 47 mg (73 ± 5 µCi) tritiated naphthalene. Samples of blood, liver, kidney, vitreous humor, lens, and cornea were taken at 16, 24, 40, 48, 70, 168, and 204 hours after exposure. The greatest uptake in most tissues was between 24 and 40 hours and lenses had high concentration of naphthalene.

The intent of the present work on the lens is twofold: (1) to establish whether petroleum, certain petroleum components, or metabolites of petroleum hydrocarbons damage the lens directly, from whatever route they enter the fish, or whether lens damage is produced indirectly as the result of damage to another system such as the kidney. (2) to prepare a reference collection of micrographs of normal lens fibers from fish indigenous to Alaskan waters to evaluate effects of damage to lenses from an oil spill, either through direct surface exposure or exposure to oiled sediments. Studies on seventeen species of fish have been completed and a manuscript which describes the comparative morphology of the normal lenses is in preparation.

EGGS AND LARVAL STAGES

Pink salmon eggs were collected, fertilized, and exposed to toluene (8-360 ppm) naphthalene (0.8-15 ppm) or water-soluble fractions of Prudhoe crude oil (0.96-9.62 ppm) by personnel at the Auke Bay NMFS laboratory. Specimens for microscopic analysis were sampled from two doses each of naphthalene and the water-soluble fraction of Prudhoe Bay crude oil at the following time sequence after exposure: 15,30, 45 sec; 1, 3, 10 min; 1, 5, 8, 24, 29 hr; 1, 2 weeks. We are examining both the surface of the egg, and the embryo as it develops, with the scanning electron microscope.

RELEVANCE TO PROBLEMS OF PETROLEUM DEVELOPMENT

The most important results of our work to date concern lens and liver changes. Naphthalene, a component of petroleum, has long been known as a causative agent in cataract formation in rabbits (van Heyingen and Pirie, 1967) and other animals (Grant, 1963). Lens damage in fish is potentially very deleterious because of possible blindness or reduced vision and obvious consequent survival difficulties. Food-chain bioaccumulation or amplification of certain petroleum components is a likely source of this type of exposure as well as for most other internal organs. Other long-term sources of petroleum contamination include sediments.

The observed liver changes parallel hepatic alterations known for other animals to be pre-neoplastic (Bannasch, 1976). Neoplasia is generally a long-term process and indications of such pathology occurring should be followed with longer term studies since this clearly pertains to the health of fish populations. Other liver alterations which involve fluctuations of lipid and glycogen on either side of normal are indicators of stress responses to the toxic materials. These changes indicate that the ability of fish to survive additional routine environmental stress may be reduced because of depletion of energy reserves or reduced liver functions.

Our newest area of research on structure of eggs and larval fish seems to be an important area in which to concentrate future efforts. If the young stages of marine fish, which generally cannot readily escape an oil slick, are as highly sensitive to petroleum as is indicated by Struhsaker et al. (1974), data on morphological changes resulting from sublethal exposures to petroleum may be extremely valuable for predicting environmental concentrations of oil which may seriously impair growth and continued survival of the young fish.

PROBLEMS ENCOUNTERED

No problems were encountered in this project.

RECOMMENDATIONS FOR FUTURE RESEARCH

Laboratory studies which utilize the data base established for the morphological changes in salmon and flatfish need to be extended to include additional species of fish and exposure conditions. For example, establishing the repeatability of alterations in liver and lens morphology under varying exposure and controlled conditions is crucial before attempting to apply these data to field studies where so many factors other than petroleum affect the fish. Structural anomalies are very clear indications of cellular changes but correlating those alterations with petroleum, on the one hand, and the ability of the fish to survive in its natural environment, on the other hand, is an infant science.

Certain components of petroleum are known carcinogens. If changes in animals showing early neoplastic responses in liver cells are not followed in long-term studies, a very clear opportunity to contribute to resolving the important issue of the degree of risk of neoplasia from increased petroleum in the marine environment will be missed.

Additionally, new studies have recently been initiated which address the problem of petroleum effects on eggs and larval fish since many Alaskan species have pelagic eggs or, in the case of salmon, may spawn in the intertidal zone, and consequently, risk contamination during embryogenesis. As we have found in earlier studies, to be most applicable, there must be a combination of carefully controlled laboratory studies and field studies in conjunction with chemical analysis of tissues and the environment for the presence of both petroleum hydrocarbons and metabolic products of the hydrocarbons.

OVERVIEW

Several perspectives are given with regard to the findings and interpretations of RU 73/74; however, the reader is referred to individual sections on "Relevance to Problems of Petroleum Development" for detailed treatment of the subject.

Results of a number of our studies indicate that many marine invertebrates, particularly larval forms, are highly susceptible to petroleum pollution. Research on the biological fate of aromatic hydrocarbons in larval invertebrates has shown that these developmental stages can substantially bioconcentrate aromatic hydrocarbons from the water column. Naphthalene, one of the principal aromatic compounds examined, not only was bioconcentrated by larval invertebrates but also underwent significant bioconversion to metabolic products. Currently, very preliminary data indicate that ppb levels of water-borne naphthalene also induce a number of morphological changes in immature forms of invertebrates. These changes may well be associated with the formation of electrophilic metabolites in the organisms. The feeding and reproductive behavior of two marine invertebrates, shrimp and nudibranchs, and the embryonic development of nudibranchs, also appear to be particularly sensitive to low levels of petroleum hydrocarbons in seawater. Extension of studies with similar experimental designs to other invertebrate species, different concentrations of oil, different temperature, and other test variables should provide valuable additional information about the effects of petroleum on critical life processes of these animals.

Our studies on the biological fate of heavy metals in arctic and subarctic marine organisms reveal that metals are bioconcentrated, and accumulate in certain organs. Further, high concentrations of metals are found within cells of these organs in subcellular fractions mediating important biochemical processes of the cell. Thus, it seems advisable to study these processes further and relate them to possible alterations in organism viability. We have shown that slight elevations in concentrations of toxic metals above levels found in seawater can alter the metabolism of aromatic hydrocarbons. This finding supports the hypothesis that increases in metals present in polluted environments, possibly arising from petroleum operations, may significantly alter the biological fate of aromatic hydrocarbons in marine organisms.

The works on the effects of water-borne petroleum on salmon homing is revealing potentially important findings. In very recent studies we have clearly been able to interrupt salmon upstream migration by introducing petroleum hydrocarbons into the "home stream" water. When the data are fully analyzed we expect to have evidence delineating the threshold concentrations of hydrocarbons that interfere with salmon homing at one or more field sites.

Our investigations of the biological fate of petroleum in subarctic and arctic marine organisms have shown that aromatic hydrocarbons are particularly accumulated in certain organs and tissues. These body areas include mucus, brain, skin, liver, gall bladder, and dark muscle of fish and thoracic segments of postlarval invertebrates. Each site, with the exception of the invertebrate thoracic segments, which has not been studied with respect to bioconversions, has thus far been identified as a prominent site for metabolite accumulation. Mucus was identified as an important route or hydrocarbon excretion. This route must now be considered in the recycling of hydrocarbons and metabolites, together with the expected elimination routes via the gills and gall bladder.

By identifying certain tissues in the body which preferentially accumulate both hydrocarbons and metabolic products, we have shown that hydrocarbon analyses of whole body tissues from field studies may lead to errors in judgment about the nature and degree of petroleum contamination of marine organisms.

Our studies with flatfish and salmon--representing subarctic demersal and pelagic species, respectively-show that each organism experiences distinctly different levels of risk from petroleum in the marine environment. Thus far we have not demonstrated that salmonid fishes are severely compromised from exposure to low to moderate levels of petroleum. Only certain parameters have been investigated thus far, however, and all studies relating to salmonid species are not yet completed. Conversely, our investigations of flatfish have provided strong indications that petroleum and other persistent marine contaminants may have substantial impact on this group of fishes. Flatfish markedly bioconcentrate petroleum from the water column and have a high capacity for converting aromatic hydrocarbons to metabolites which are stored in certain tissues in amounts often far exceeding the hydrocarbons themselves. Notable areas of metabolite storage in flatfish are the liver and skin, both of which have been shown to undergo various pathological changes, including neoplasia. In fact, we and others have found that skin tumors of flatfish exist in near epizootic proportions in certain areaa, although there is no clear relation betwen incidence of these lesions and pollution, and the causes of these tumors are, in fact, unknown. We have found, however, that a high percentage (~90% of certain species of flatfish taken from a polluted estuary near Seattle exhibited substantial pathological changes (hepatoma, fin erosion), which were either non-existent or found in low incidence in the same species from less urban areas. It is also noteworthy that in our studies in which English sole were held in oil-contaminated sediment for up to five months, these fish developed a number of pathological alterations. An additional observation which bodes unfavorably for flatfish is that hydrocarbons in sediments have been shown to reach very high levels after oil contamination resulting from long-term discharges into the environment, thereby providing the possibility for severe exposure which may lead to the kinds of biochemical, physiological, and pathological alterations we have observed.

Because of the persuasive body of evidence given above on the high risk status of flatfish, it is our intention in future studies of marine fish to emphasize research on effects of petroleum on the pleuronectids. These fish are, like salmon, of great economic importance and are widely used as food. Results of this research will be compared with similar studies on salmonids and other marine species that are not closely related to the pleuronectids. That is to say, our data base will be significantly broadened to allow us to obtain a better phylogenetic perspective of effects of oil development on arctic and subarctic marine environments.

In conclusion, we re-emphasize, in addition to the undeniable importance of effects studies, the importance and timeliness of studying the biological fates of high molecular weight aromatic hydrocarbons and products of biological and chemical oxidation. Studies of the retention and toxicity of hydrocarbon metabolites and the potential for food-web transfer of these structures should be included in future work.

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4. TRANSPORT, RETENTION, AND EFFECTS OF THE WATER-SOLUBLE FRACTION OF COOK INLET CRUDE OIL IN EXPERIMENTAL FOOD CHAINS (RU 389)

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OBJECTIVES

The objectives of this study are to determine the accumulation and passage of petroleum constituents in experimental marine food chains and the effects of petroleum exposure on the test organisms. The specific objectives are:

1. To determine the accumulation, retention and transfer of petroleum constituents in experimental food chains consisting of up to four trophic levels including primary producers, consumers and primary and secondary carnivores. Exposure will be either from water-soluble fractions of petroleum accumulation from food and water exposure pathways. Adult and larvae food chains will be studied.

2. To determine mortality of experimental animals under various exposure conditions incluing mortality in eggs and larvae from exposed adults.

3. To determine morphological and behavioral abnormalities caused by selected exposure conditions.

4. To predict potential effects of crude oil on populations and communities in terms of reproductive success, energy utilization and growth.

5. To recommend maximum allowable levels of petroleum components in the water column and fish food organisms, and if possible, to identify components of crude oil with the greatest potential for adverse biological effects.

METHODS

APPARATUS

All testing was made under continuous-flow conditions using a dosing apparatus developed by project personnel (see Benville et al., manuscript in preparation). This apparatus (Figure 1) produced a stable

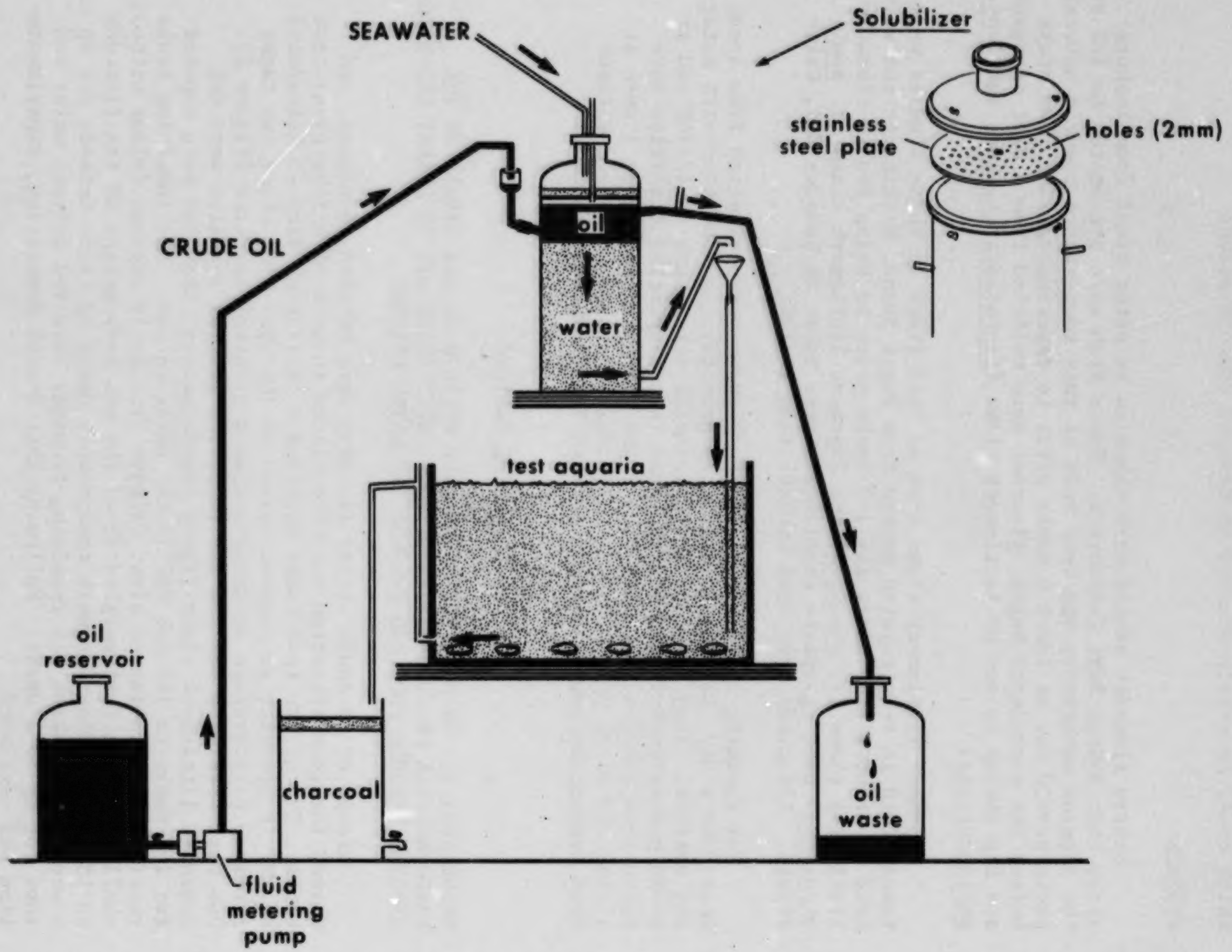

Figure 1. Apparatus for continuously dosing flounder or clams to the water-soluble fraction of crude oil.

outflow of the water-soluble fraction (WSF) of Cook Inlet crude oil which could be easily diluted to desired concentrations.

ANIMALS

Starry flounder adults were taken in an otter trawl from inshore areas near Bodega Bay, California. These fish were acclimated to 12C at the Tiburon Laboratory and were held at this temperature under a natural photo-period for at least 4 weeks prior to experimentation. Two weeks before the experiment began, flounder were switched from a diet of squid and Bay shrimp to one of littleneck clams (*Protothaca staminea* and *Tapes semidecussata*).

Japanese littleneck clams used as food items in these studies were transported in refrigerated trucks from Puget Sound, Washington and were held at Tiburon Laboratory for 1-2 weeks prior to being fed to flounder. Littleneck clams (*P. staminea*) and Japanese littleneck clams (*T. semidecussata*) used in uptake experiments were taken in Tomales Bay, California. All clams were held without food at 12C.

For spawning experiments, adult flounder were collected from areas near Bodega Bay, California, while in pre-spawning condition with maturing ovaries. They were acclimated briefly for 1 week at 12-14C and at 29-30 ppt salinity. Littleneck clams in pre-spawning condition were taken from Tomales Bay, California, and were acclimated for 1 week at 17-18C and 31-32 ppt salinity. Flounder and clams were held without food during the 1-week acclimation and 7-day exposure periods.

EXPERIMENTAL DESIGNS

EXPERIMENT 1: UPTAKE, RETENTION, AND EFFECTS OF LOW LEVELS OF THE WATER-SOLUBLE FRACTION (WSF) OF COOK INLET CRUDE OIL TO STARRY FLOUNDER EXPOSED THROUGH FOOD AND THROUGH THE WATER COLUMN

Groups of 30 adult starry flounder were weighed, measured, and marked for identification and were placed in each of 4 fiberglass test tanks. Two of the test tanks received a continuous flow (6 liter/min) of WSF in seawater at concentrations of 100 ppb. The other two tanks received 6 liters/min of uncontaminated (control seawater (Figure 2). One tank receiving WSF and one receiving control seawater were fed Japanese littleneck clams (*Tapes semidecussata*) that had been exposed for 1-2 weeks to 100 ppb WSF in the water column. The remaining tanks received uncontaminated clams (Figure 2). Daily rations (clams without shells) to each tank weighed 4% of the wet body weight of the flounder in the tank. Flounder were continously dosed by these methods for up to 5 weeks after which all remaining flounder received control water and food for up to 2 weeks. Following this 2-week depuration, experimentation was concluded.

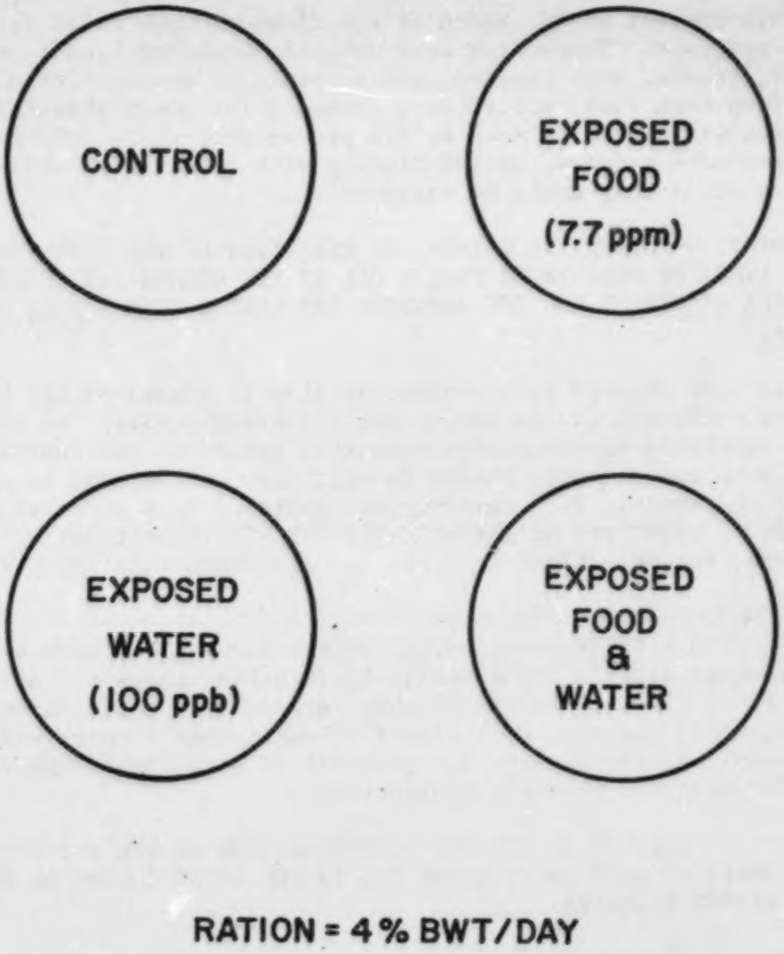

Figure 2. Dosing scheme for exposing starry flounder to the water-soluble fraction of crude oil in water, food, or water and food.

Water samples were taken and analyzed daily (gas chromatography) to determine the concentration of WSF in test tanks and in the solubilizer bottles feeding into the tanks. Every day temperature and salinity were measured and subsamples of clams fed to flounder were taken for analysis of petroleum hydrocarbons.

On alternating weeks, three or six flounder were taken from each tank for analysis. These fish were weighed, measured, and identified. The liver, gonads, gall bladder, and a sample of muscle tissue were removed from each fish; after being examined for gross abnormalities, each tissue was placed in each of two pre-weighed glass culture tubes. These tubes were weighed, sealed tightly with teflon-line screw caps, and frozen until they could be analyzed.

EXPERIMENT 2: COMPARATIVE UPTAKE AND RETENTION OF THE WATER-SOLUBLE FRACTION (WSF) OF COOK INLET CRUISE OIL BY THE COMMON LITTLENECK CLAM (PROTOTHACA STAMINEA) AND THE JAPANESE LITTLENECK CLAM (TAPES SEMIDECUSSATA)

Clams were exposed to a continuous flow (1 ℓ/min) of the WSF of Cook Inlet crude oil at 100 ppb in two 133-liter aquaria; an additional aquarium receiving uncontaminated seawater served as the control. Sixty clams of each species were placed in each tank and exposed to the WSF for up to two weeks. This exposure was followed by a one-week depuration period. Water was monitored daily for WSF concentration, salinity, temperature, and pH. Clams were sampled from each tank every three days.

Whole clam tissues were pooled, if necessary, from each sampling to make up 10-gram samples for aromatic-hydrocarbon analysis. All samples were placed in pre-weighed glass tubes, sealed with teflon-lined caps, and frozen until the time of analysis. Four tissue samples were taken at each sampling, two samples for analysis of monoaromatic-hydrocarbons and two for dicyclic aromatic hydrocarbons.

EXPERIMENT 3: EFFECTS OF CHRONIC CONCENTRATIONS OF THE WATER-SOLUBLE FRACTION (WSF) OF COOK INLET CRUDE OIL IN THE WATER COLUMN ON PRE-SPAWNING STARRY FLOUNDER

Groups of 20 adult starry flounder were placed in each of 2 fiberglass tanks. One tank received a continuous flow (7 liters/min of WSF in seawater at a mean concentration of 120 ppb. The other tank received uncontaminated (control) seawater at the same flow rate. The system for exposure was similar to that described in Experiment 1. Since flounder rarely feed during spawning season, no food was given during exposure.

Flounder were continuously dosed for 8 days; flounder not taken for analysis during this period were allowed to depurate for 1 week after cessation of exposure. Water samples were taken and analyzed daily to determine the concentration of monoaromatics in the WSF of the tanks and solubilizer effluent. Temperature, salinity, oxygen, and flow rates were monitored daily. Mean temperature was 13.5C; mean salinity was 29 ppt; oxygen concentration was between 7.0 and 8.7 ppm.

Two flounder were taken from each tank daily for analysis. These fish were weighed, measured, and autopsied. The ovaries, testes, liver and gall bladder were removed. Gonads were weighed, measured and examined microscopically for abnormalities such as presence of dead eggs or immotile sperm.

Maturing ovaries were examined microscopically to determine developmental stage, presence of opaque dead or decaying eggs, and the gross appearance (color and deliquescence). Maximum egg diameters of 10 eggs from the ovary of each female were measured. Spermatozoa from ripe males were examined under the microscope for motility.

The ventral gonads, the gall bladder and half of the liver were immediately frozen for petroleum hydrocarbon analysis. The dorsal gonad and remaining half of the liver were preserved for histological examination. Tissues were prepared for histological examination by making frozen sections and staining for lipids with Oil Red O according to the technique in Humason (1972).

EXPERIMENTS 4A, 4B, 4C: EFFECTS OF CHRONIC CONCENTRATIONS OF THE WATER-SOLUBLE FRACTION (WSF) OF COOK INLET CRUDE OIL IN THE WATER COLUMN ON SPAWNING STAGES OF LITTLENECK CLAMS

Groups of 40 adult common littleneck clams were placed in each of 3 glass tanks. Each tank received a continuous flow (mean = 2 liters/min) of seawater. Two tanks were continuously dosed with mean concentrations of 90 and 100 ppb WSF in seawater; the third tank was a control. The exposure system was similar to that described in Experiment 2. Clams were not fed during exposure.

Clams were continuously dosed for up to 4 days; clams not removed for analysis during this period were allowed to depurate for up to 1 week after cessation of exposure. Water samples were taken and analyzed daily to determine concentration of aromatics in the WSF of the tanks and solubilizer effluent. Mean temperature was 17.5C; mean salinity was 32 ppt; oxygen concentration fluctuated between 9.10 and 10.0 ppm.

Four clams were taken from each tank daily. Clams were measured and autopsied. Gonads were cut open and a sample of eggs or sperm was removed for microscopic examination in ambient seawater. The entire body of the clam was removed, placed in pre-weighed test tubes, and frozen for analysis of tissue concentrations of aromatics. Other subsamples were taken daily and preserved for histological examination. Maturing eggs were examined under the compound microscope to determine developmental stage, presence of abnormal or dead eggs, and maximum diameter of 10 eggs from each female was measured. Spermatozoa were examined for motility.

ANALYTICAL PROCEDURES

ANALYSIS OF WATER SAMPLES

Water samples taken daily from exposure tanks were analyzed for monocyclic-aromatic hydrocarbons by extracting one liter twice with 10 ml of TF-Freon and injecting 3.2 microliters of each extract into the gas chromatograph (GC) (column packed with Bentone 34 on chromosorb PAW). Liter samples taken daily from solubilizer effluent flowing into the exposure tanks were extracted 3 times with 10 ml of TF-Freon and injected into the GC.

In addition, one-liter solubilizer samples were extracted with 80 ml methylene chloride for analysis of dicyclic aromatic compounds. Extracts were concentrated to about 10 ml and stored in a freezer. Subsequent preparation for analysis followed procedures of MacLeod et al. 1977.

ANALYSIS OF TISSUE SAMPLES

Samples analyzed for dicyclic-aromatic hydrocarbons and aliphatic compounds were processed according to MacLeod et al. 1977. Samples were analyzed for low-boiling aromatic hydrocarbons using procedures developed by project personnel for these experiments (see Table 1 for an outline of these methods).

ADDITIONAL ANALYSES

Through a subcontract to the National Analytical Facility, a number of analyses of water, tissue, and crude oil samples were made by gas chromatography (GC) or mass spectrometry (MS) to verify results obtained by in-house analysis and to positively identify unknown hydrocarbons in tissue and water extracts.

RESULTS

EXPERIMENT 1: UPTAKE, RETENTION, AND EFFECTS OF LOW LEVELS OF THE WATER-SOLUBLE FRACTION (WSF) OF LOWER COOK INLET CRUDE OIL TO STARRY FLOUNDER EXPOSED THROUGH FOOD AND THROUGH THE WATER COLUMN

Starry flounder were exposed to WSF of Cook Inlet crude oil in water, food, or water and food for up to 5 weeks. Concentrations of low-boiling-point aromatic compounds averaged 100 ppb in exposed test tanks while WSF-contaminated food averaged nearly 8 ppm (Table 2). Concentrations of napthalenes in exposed-water tanks were less than 10 ppb, but were roughly 40 times higher in clam tissue used as WSF-contaminated food.

To date, no tissues other than liver have been analyzed in sufficient numbers to make a meaningful presentation. Seventy livers were analyzed for low-boiling-point aromatic components and 18 were analyzed for higher-boiling-point (dicyclic) aromatic components.

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Table 1.

Procedure for analysis of low-boiling-point hydrocarbons in animal tissues.

1. Place 10 grams of tissue in a clean, glass culture tube (with a teflon-seal screw cap).
2. Add 6 ml of 4N NaOH and 4 ml of TF-Freon and cap tightly.
3. Place tube in oven (or water bath) for 18 hours at 30C. Shake tube 4 or 5 times during this time.
4. Remove tube from oven and shake vigorously for one minute.
5. Centrifuge tube (while still warm) for 10 minutes at 3000 rpm.
6. If freon layer is clear (not cloudy), draw off with pipette and store in a clean, glass vial with a teflon-seal screw cap until ready to inject on GC.

Emulsions:

If the freon layer is clouded, use following procedure.

7. Freeze clouded sample and recentrifuge while still frozen (20-30 minutes at 2000-3000 rpm).
8. If sample is still cloudy, repeat.
9. If sample has not cleared, add 1-2 ml of 20% H_2SO_4, shake, and recentrifuge. A 15-20% reduction in recovery will result if it is necessary to follow this step.

RECOVERY RATE = 90-96% if tubes remain tightly capped.

TABLE 2. Estimated concentrations of monocyclic aromatic hydrocarbons and other low-boiling-point hydrocarbons.

	CONCENTRATION (PPM)							
SAMPLE	BENZENE	TOLUENE	ETHYL-BENZENE	P-XYLENE	M-XYLENE	O-XYLENE	OTHER	TOTAL
SEAWATER	0.041	0.043	0.002	0.00005	0.007	0.004	<0.01	0.100
CLAM	2.23	1.43	<0.04	<0.04	0.30	<0.04	3.74	7.70

Uptake of Hydrocarbons from Food

Flounder receiving contaminated food accumulated low-boiling-point compounds during exposure, reaching an average concentration of 1.72 ppm after 5 weeks (Table 3). None of the compounds detected by GC analysis have been identified; we suspect they were metabolites (Figure 3). It is curious that no monocyclic-aromatic compounds were detected in livers from this treatment group because those compounds were detected in substantial amounts in clam tissue used as food (Figure 4 and Table 2).

Napthalene concentrations in flounder livers were not significantly different from estimated concentrations in the clams. Levels of napthalene, 2-methylnapthalene, and 1-methylnapthalene in livers averaged 95, 82, and 54 ppb, respectively.

Uptake of Hydrocarbons from Water

After 2 weeks of exposure, the mean concentration of monocyclic-aromatic compounds was 22 ppm in flounder livers (Table 3). After 5 weeks exposure, the mean level was 49 ppm. The concentration of cyclohexanes and unidentified components (compounds not also found in chromatograms of livers from control flounder) reached an average level of 131 ppm. The total concentration of low-boiling-point compounds averaged 180 ppm. In one flounder liver, this concentration exceeded 300 ppm (Figure 5).

The liver of one flounder exposed for only one week to 100 ppb WSF contained nearly 150 ppm of monocyclic aromatic compounds, This flounder had lost weight, was in poor shape (a condition not attributed to the WSF exposure), and as a result, may not have been metabolizing hydrocarbons as efficiently as healthy flounder. However, the tissue concentrations were noteworthy because they represented remarkable accumulation of petroleum hydrocarbrons. After one week, concentrations of monocyclic aromatics in the liver were between 860 (benzene) and 97,250 (p-xylene) times higher than the concentration in the water column.

Concentrations of napthalene, 2-methylnapthalene, and 1-methylnapthalene reached maximum levels of 4.6, 4.5, and 3.4 ppm after 5 weeks exposure. These levels represent accumulation factors of 790, 1700, and 1480 times the water concentration.

Uptake of Hydrocarbons from Water and Food

No significant differences were found between concentrations of monocyclic aromatics after 1-week and 5-weeks exposure (Table 3). However, the concentration of cyclohexanes and unidentified low-boiling compounds increased continually during the exposure period. Chromatograms of livers from this exposure group were virtually identical to those from the water-exposure group with regard to kinds and numbers of compounds and relative concentration of components (Figure 6).

TABLE 3. Concentrations of monocyclic aromatic and other low-boiling-point hydrocarbons in livers of starry flounder exposed to the WSF of Cook Inlet crude oil.

SOURCE OF EXPOSURE	LENGTH OF EXPOSURE (Weeks)	CONCENTRATION (PPM)							
		BENZENE	TOLUENE	ETHYL-BENZENE	P-XYLENE	M-XYLENE	O-XYLENE	OTHER	TOTAL
FOOD	1	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	0.39	0.39
	3	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	0.63	0.63
	5	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	1.72	1.72
WATER	2	3.09	9.50	2.15	0.93	4.44	1.94	131.88	153.93
	5	6.11	18.35	6.35	1.81	9.71	6.64	131.11	180.08
FOOD & WATER	1	5.61	17.01	3.95	1.16	7.76	5.50	64.04	105.03
	2	4.11	13.21	3.38	0.83	4.84	1.68	80.53	108.58
	5	6.54	14.28	5.45	1.30	8.85	6.13	198.68	241.23

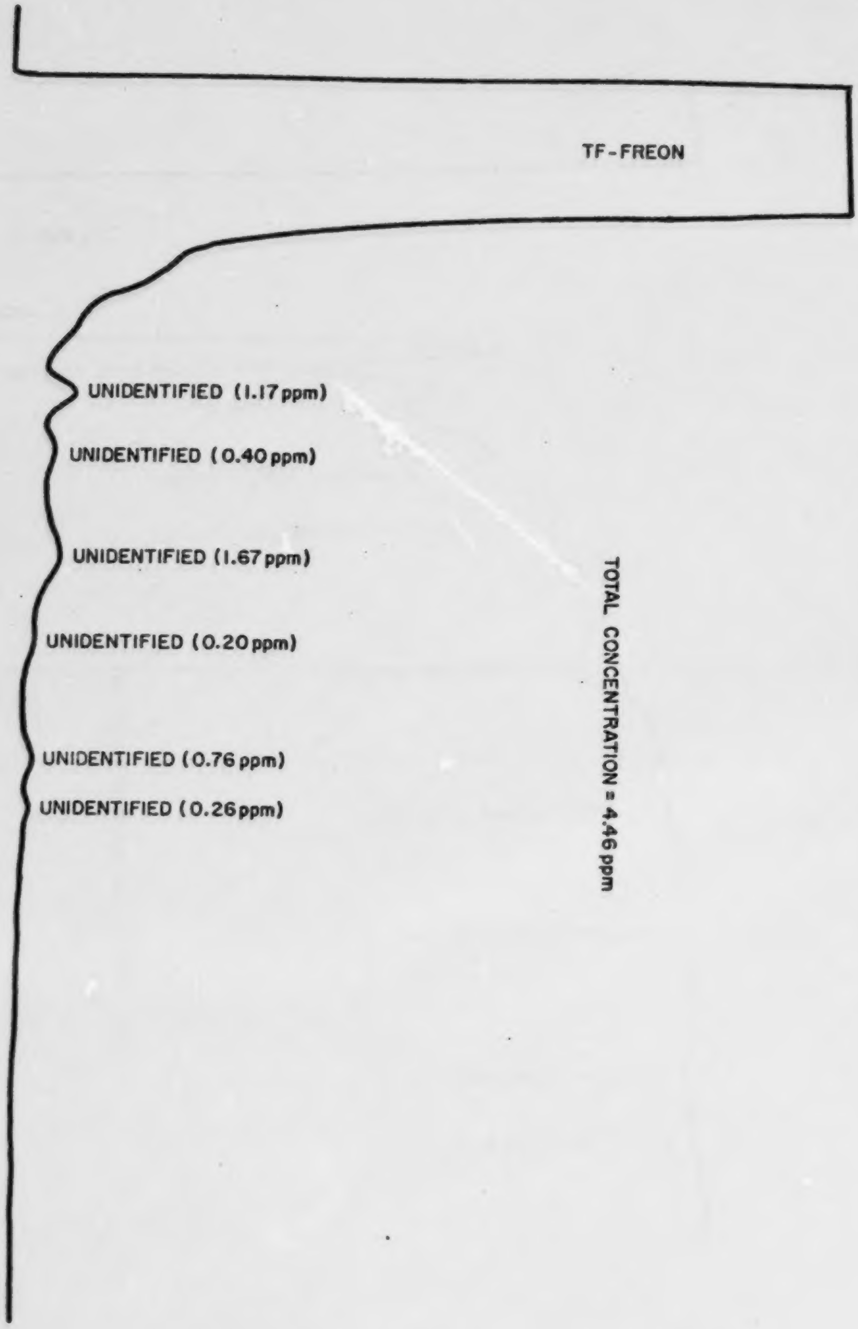

Figure 3. Chromatogram of low-boiling hydrocarbons detected in the liver of a starry flounder fed WSF-contaminated clams for 5 weeks.

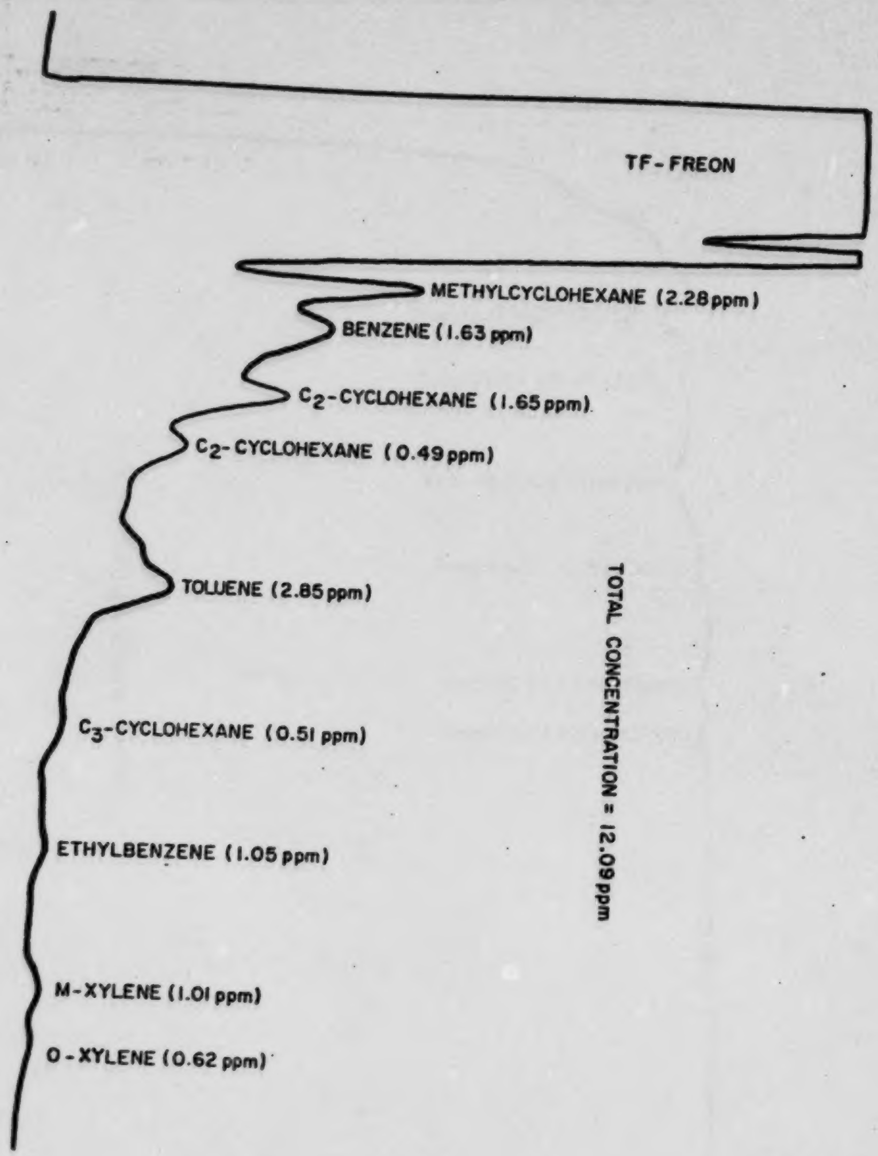

Figure 4. Chromatogram of low-boiling hydrocarbons in a sample of littleneck clams exposed for 1-2 weeks to 100 ppb WSF. This sample had the highest concentration of hydrocarbons of any sample analyzed.

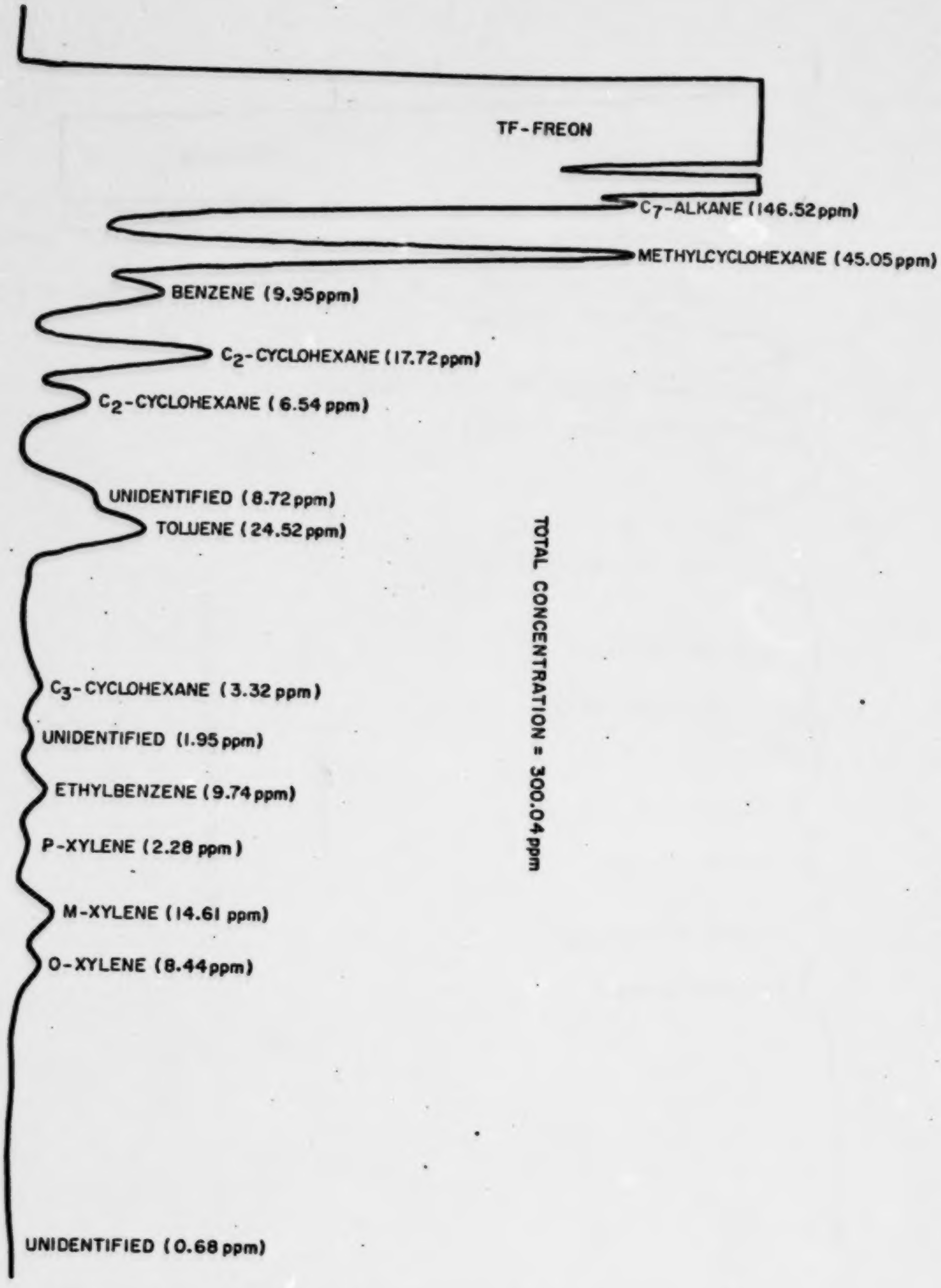

Figure 5. Chromatogram of low-boiling hydrocarbons detected in the liver of a starry flounder exposed 5 weeks to 100 ppb WSF in the water column.

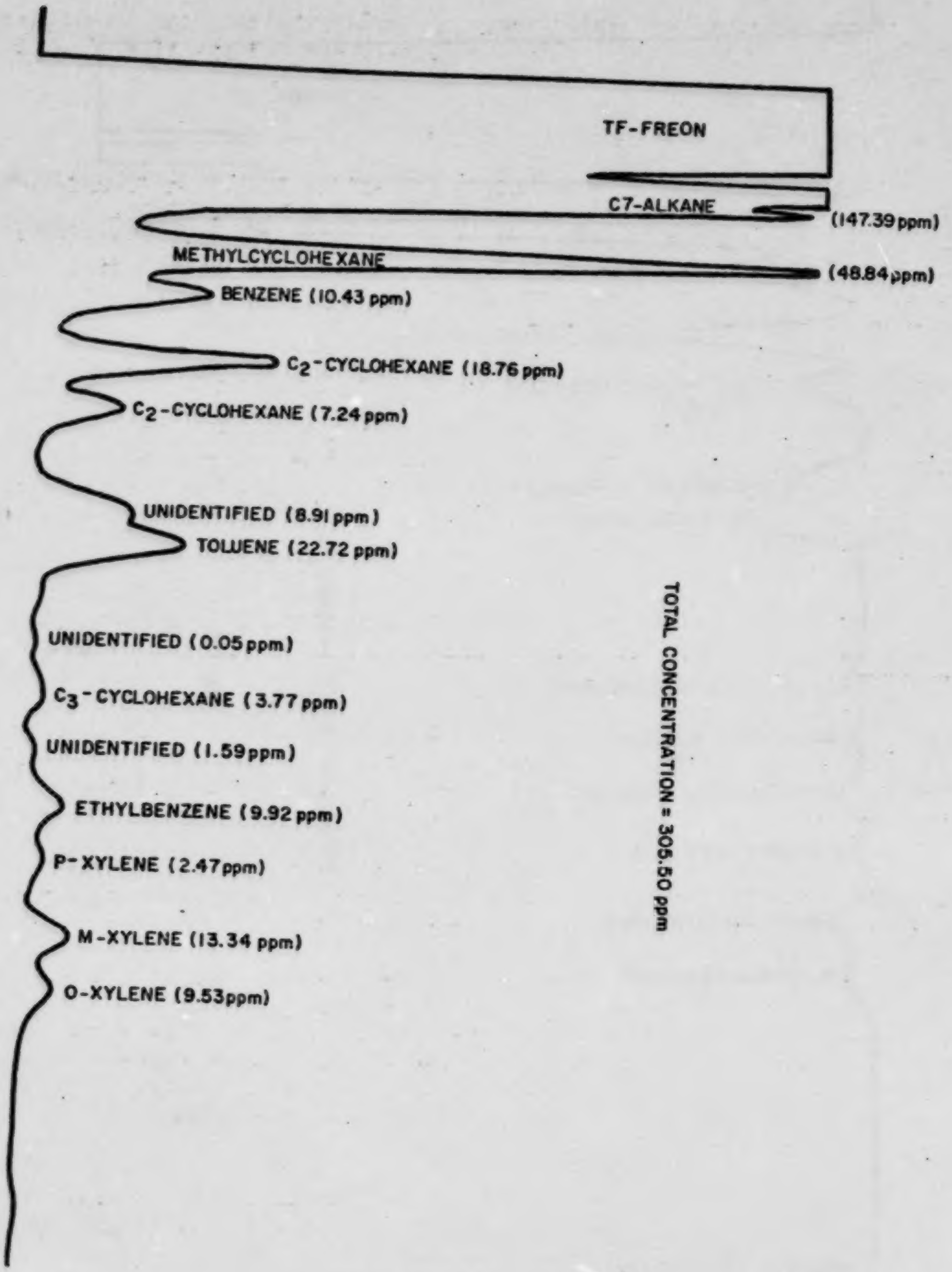

Figure 6. Chromatogram of low-boiling hydrocarbons detected in the liver of a starry flounder exposed to 100 ppb WSF in the water column and fed WSF-contaminated clams for 5 weeks.

Concentrations of napthalene, 2-methylnapthalene, and 1-methylnaphthalene reached maximum levels of 4.3, 4.2, and 4.3 ppm after 5-weeks exposure.

Retention of Low-Boiling-Point Components

Monocyclic-aromatic compounds were rapidly lost from flounder livers following cessation of exposure to WSF. Within 48 hours, most of these six compounds had dropped to near or below detectable limits (less than 40 ppb). After 2 weeks, only cyclohexanes and unidentified compounds were detected in the low-boiling-point range, at concentrations far below maximum levels (Table 4).

Growth Rates of Flounder in Exposure and Control Groups

Flounder grew well under all treatment conditions. No treatment group grew at rates significantly different from controls or other treatment groups. Test flounder remaining at the end of the exposure period had grown an average 4.6% (length) and were 19.5% heavier than at the start of the experiment.

EXPERIMENT 2: COMPARATIVE UPTAKE AND RETENTION OF THE WATER-SOLUBLE FRACTION (WSF) OF COOK INLET CRUDE OIL BY THE COMMON LITTLENECK CLAM (*PROTOTHACA STAMINEA*) AND THE JAPANESE LITTLENECK CLAM (*TAPES SEMIDECUSSATA*).

To date, 18 of 120 samples have been analyzed for monocyclic aromatics; none of 120 dicyclic-aromatic samples have been analyzed. As such, the results given should be viewed as preliminary indicators of the magnitudes of accumulation of monocyclic-aromatic hydrocarbons.

The average water concentration of WSF (Table 5) in exposed tanks was 140 ppb. The concentration of water-soluble hydrocarbons from the solubilizer bottle averaged 1.43 ppm (Table 5).

After 5 days of exposure, the average tissue concentration of monocyclic-aromatic hydrocarbons was 0.987 ppm (μg/g wet weight), a biomagnification 14 times higher than the concentration in water (Table 5). Following 12 days of exposure, the average tissue concentration of 1.77 ppm (μg/g) in *Tapes* was 25 times higher than the water concentration. Clams allowed to depurate for 6 days contained less than 0.08 ppm (μg/g) monoaromatics.

The average tissue concentration in *Protothaca* was 0.60 ppm after an exposure of 7 days. This mean concentration was four times higher than that in the water column (Table 5).

Table 4.

Mean concentrations of monocyclic aromatic and other low-boiling-point hydrocarbons in livers of starry flounder exposed to the water-soluble fraction of Cook Inlet crude oil.for 5 weeks and then allowed to depurate.

SOURCE OF EXPOSURE	LENGTH OF DEPURATION (days)	CONCENTRATION (PPM)							
		BENZENE	TOLUENE	ETHYL-BENZENE	p-XYLENE	m-XYLENE	o-XYLENE	OTHER	TOTAL
FOOD	2	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	2.46	2.46
	14	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	0.50	0.50
WATER	2	<0.04	<0.04	0.17	<0.04	0.59	0.04	77.68	78.48
	7	<0.04	<0.04	<0.04	<0.04	0.04	0.05	11.98	12.07
	14	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	5.19	5.19
FOOD & WATER	2	<0.04	<0.04	0.63	<0.04	0.82	<0.04	85.44	86.89
	7	<0.04	<0.04	<0.04	0.04	0.44	<0.04	20.06	20.54
	14	<0.04	<0.04	<0.04	<0.04	<0.04	0.04	9.69	9.73

TABLE 5. Concentrations of monocyclic aromatic hydrocarbons in gonads of starry flounder exposed to the WSF of Cook Inlet crude oil prior to spawning.

SAMPLE	TIME (Day)	STAGE	CONCENTRATION (PPM) - µg/g or µl/l (wet)								
			BENZENE	TOLUENE	ETHYL-BENZENE	P-XYLENE	M-XYLENE	O-XYLENE	TOTAL	ACCUMULATION	DEAD EGGS
SEAWATER	1	------	0.090	0.074	0.005	ND	0.007	0.006	0.182		
TISSUES	1										
CONTROL M		RIPE	ND	ND	ND	ND	ND	ND	ND		
CONTROL F		IMMATURE	ND	ND	ND	ND	ND	ND	ND		NO
CONTROL F		MATURE	ND	0.052	0.020	ND	ND	ND	ND		NO
EXPOSED F		IMMATURE	0.309	ND	0.011	ND	ND	ND	0.320	1.6X	NO
SEAWATER	2	------	0.046	0.060	0.004	ND	0.007	0.006	0.123		
TISSUES	2										
EXPOSED F		MATURE	1.633	1.873	ND	ND	ND	ND	3.506	28.5X	NO
SEAWATER	3	------	0.045	0.059	0.004	ND	0.006	0.005	0.119		
TISSUES	3										
EXPOSED M		RIPE	ND	ND	ND	ND	ND	ND	ND		
EXPOSED F		IMMATURE	ND	ND	ND	ND	ND	ND	ND		NO
SEAWATER	4	------	0.032	0.048	ND	ND	0.005	0.003	0.088		
TISSUES	4										
EXPOSED M		RIPE	ND	ND	ND	ND	ND	ND	ND		
EXPOSED F		MATURE	2.878	8.334	1.740	0.726	3.922	3.262	20.862	235.9X	YES
SEAWATER	5	------	0.041	0.053	0.004	ND	0.006	ND	0.104		
TISSUES	5										
EXPOSED M		RIPE	ND	0.010	ND	ND	ND	ND	0.010		
EXPOSED F		MATURE	1.554	2.846	0.400	ND	0.851	0.069	5.720	55.0X	YES
SEAWATER	6	------	0.046	0.057	0.004	ND	0.005	ND	0.112		
TISSUES	6										
EXPOSED F		MATURE	1.539	2.892	ND	ND	0.612	ND	5.043	45.0X	YES
EXPOSED F		MATURE	1.378	2.495	ND	ND	0.255	0.008	4.136	36.9X	YES
SEAWATER	7	------	0.026	0.044	ND	ND	0.005	ND	0.075		
TISSUES	7										
EXPOSED F		MATURE	1.254	2.290	ND	0.007	0.845	ND	4.396	58.6X	YES
EXPOSED F		MATURE	1.038	2.916	0.518	0.050	0.513	0.227	5.262	70.1X	YES

ND= Not detectable, but may be present. M= Male; F= Female. MATURE = Maturing, but not running ripe. IMMATURE = Not maturing for spawning in this year.

EXPERIMENT 3: EFFECTS OF CHRONIC CONCENTRATIONS OF THE WATER-SOLUBLE FRACTION (WSF) OF COOK INLET CRUDE OIL ON PRE-SPAWNING STARRY FLOUNDER.

Starry flounder in pre-spawning condition were exposed to WSF for 1 week. Female flounder were not fully ripe; male flounder were ripe with motile spermatozoa. Concentrations of monoaromatic components averaged 117 ppb in exposed tanks (Table 6); analysis of water concentrations of other aromatic compounds is incomplete. Although four tissues were taken from each fish for analysis, to date, only ovaries and testes have been fully analyzed.

Uptake of Hydrocarbons from Water

After 1 week of exposure, the average concentration of monocyclic aromatics in mature ovaries average 5.26 ppm (range 3.50 to 20.8 ppm). In females with immature (not ripening) ovaries, monocyclics were low or undetectable. Generally, the riper the eggs, the higher the accumulation of monocyclics. The ripest female had a maximum accumulation of 235.9 X the water concentration of monoaromatics (Table 6); Toluene accumulated to higher concentrations than any of the other monoaromatics. Testes contained only traces of monocyclic aromatics (Table 6).

Analysis of samples for other aromatics is incomplete, although samples analyzed by the National Analytical Facility showed a rapid uptake of these compounds in ovaries. At the end of the 7-day exposure period, a maximum 9.4 ppm (μg/g; dry) total napthalenes had accumulated in the ovaries of one female.

Effects on Adults and Gonads

No motality occurred among adults. Females were observed to be stressed as indicated by faster and more irregular ventilation rates. Dead, opague white eggs were observed in ovaries by day 4 (Table 5). Ovaires were pale, possibly due to effects on carotenoid pigments or to destruction of red blood cells leading to pale appearance of capillaries of the ovarian membrane. This observation will be investigated further. No obvious effect on testes or on sperm motility was observed.

Preliminary histogological examination of eggs and ovarian tissue revealed necrosis of eggs and altered distributions of lipids in the eggs. These examinations are continuing.

Because eggs were not fully mature, no spawning occurred in the tanks, and artificial fertilization was not possible. Additional experiments on spawning adult flounder are planned for this winter (1978).

Table 6. Concentrations of monocyclic-aromatic hydrocarbons in whole clam tissue samples (1-2 clams per sample) of common littleneck clams (*Protothaca staminea*) exposed to 93 ppb of the water-soluble fraction of Cook Inlet crude oil prior to spawning.

			CONCENTRATION (ppm or ug/g-wet weight)						
SAMPLE	DAYS EXPOSED	N	BENZENE	TOLUENE	ETHYL-BENZENE	P-XYLENE & M-XYLENE	O-XYLENE	TOTAL	ACCUMULATION*
Control male	1	2	ND**	ND	ND	ND	ND	ND	-
Exposed female		2	0.414	0.749	ND	0.093	0.039	1.295	13.6
Exposed female		1	0.306	0.443	ND	ND	ND	0.749	7.1
Control female	2	2	ND	ND	ND	ND	ND	ND	-
Exposed female		2	0.639	ND	ND	0.073	ND	0.712	7.4
Exposed male		2	0.498	ND	ND	ND	ND	0.498	5.2
Exposed female	3	1	0.447	0.879	0.011	0.004	0.044	1.385	15.2
Control male	4	2	ND	ND	ND	ND	ND	ND	-
Exposed female		1	0.705	1.418	0.387	ND	ND	2.510	27.9
Exposed male		2	ND	0.661	0.042	0.011	ND	0.714	7.9
Exposed male		1	ND	0.363	ND	ND	ND	0.363	4.0

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* ACCUMULATION = Tissue concentration/water-column concentration

** Not detectable

EXPERIMENT 4A, 4B, and 4C: EFFECTS OF CHRONIC CONCENTRATIONS OF THE WATER-SOLUBLE FRACTION (WSF) OF COOK INLET CRUDE OIL IN THE WATER COLUMN ON SPAWNING STAGES OF LITTLENECK CLAMS.

(Although several experiments were conducted with Japanese and common littleneck clams, this report includes only results from the exposure of pre-spawning common littleneck clams; analysis of results from other experiments is incomplete.)

Common littleneck clams were exposed for 4 days prior to spawning. Female clams were not fully mature, but male clams were ripe with motile spermatozoa. Concentrations of monoaromatic components in the water column average 93 ppb (μl/l). Analysis of water concentrations of other aromatic compounds is incomplete. To date, whole tissue samples of exposed clams have been completed; tissues of clams allowed to depurate 1 week after exposure are still being analyzed. Observations of dead eggs and spermatozoa were made on freshly-dissected clams after the 1-week depuration; histological examination of these clams is underway.

Uptake of Hydrocarbons from Water

After 4 days of exposure, the average concentration of monocyclic aromatics in whole clam tissue was 1.20 ppm (μg/g wet weight), and ranged from 0.36 to 2.51 ppm (Table 6). Highest accumulations occurred in female clams. Generally, the riper the eggs in the female, the higher the accumulation of monocyclics. The ripest female had tissue concentrations of monoaromatics that were 28 times higher than those in the water column (Table 6).

Samples analyzed by the National Analytical Facility showed that clams rapidly accumulated dicyclic-aromatic hydrocarbons. After a 4-day exposure, a maximum of 0.60 ppm total napthalenes was detected in female clams.

Effects on Adults and Gonads

No mortality occurred in adult clams, although adults were observed to have weakened adductor muscles following exposure. No mortality of eggs or spermatozoa was observed in clams autopsied during the exposure period. After 1 week of depuration, however, dead eggs and spermatozoa were found in the gonads of exposed clams; there appears to be a delayed effect of WSF exposure on clam gametes. Since eggs were not fully mature, no spawning was induced and artificial fertilization was impossible. Additional experiments with ripe adults are planned.

Preliminary analyses of results from similar experiments with Japanese littleneck clams indicate that the common littleneck is more sensitive to petroleum hydrocarbons, even though uptake of monocyclic aromatics in Japanese littleneck clams is of the same order or magnitude as in common littlenecks.

PRELIMINARY INTERPRETATION OF RESULTS

EXPERIMENT 1

Flounder accumulate low-boiling-point compounds much more rapidly through the water column than they do through molluscan food itmes. Monocyclic-aromatic hydrocarbons are either metabolized into other compounds during digestive uptake or are not accumulated through that pathway.

Concentrations of monocyclic aromtics in flounder liver were maximum within a week, yet concentrations of cyclohexanes and unidentified components increased throughout the exposure period. The cyclohexanes and unidentified compounds were below detectable levels in the water column and in control tissue samples.

Flounder were not visibly affected physiologically or behaviorally by the chronic exposures to which they were subjected. However, we would not rule out adverse cellular or subcellular effects; we did not attempt to evaluate such effects in this experiment.

It was unclear whether flounder had accumulated maximum concentrations of petroleum hydrocarbons (and metabolites) after 5 weeks of exposure. Further analyses of tissues for monocyclic and dicyclic compounds must be made before further conclusions can be drawn.

EXPERIMENT 2

Concentrations of petroleum hydrocarbons in *Tapes* did not reach maximum levels in two-week exposures. Further analyses are needed to determine levels reached in *Protothaca* tissues. At low concentrations of WSF, it is probable that long-term exposures are necessary to reach maximum tissue accumulation; in littleneck clams, maximum tissue concentrations may be independent of water concentration.

For identical exposures, *Tapes* accumulated aromatic hydrocarbons to concentrations 3 times that found in *Protothaca*. Sublethal effects resulting from this differential uptake could have consequences to population structure and distribution of these clam species, since the species occupy similar habitats and are often sympatric.

EXPERIMENT 3

Starry flounder in pre-spawning condition accumulated monocyclic aromatics from 100 to 1000 times the concentrations in water. Whether these accumulations represented maxima was not certain; tissue concentrations at this exposure level increased during the first few days of exposure after which there was no significant change (Table 5). The alkyl-cyclohexanes and unidentified compounds discussed in Experiment 1 also appeared in ovary samples. Further work is in progress on these compounds.

Flounder were affected by short exposures to low concentrations of the WSF of Cook Inlet crude oil; egg death occurred in ovaries within 1 week of exposure. Other sublethal effects are probable. As flounder used in this experiment were about a month from spawning, it is likely that stages closer to spawning will exhibit even greater accumulation and effects from exposure.

PROBLEMS ENCOUNTERED

Many compounds were present in water samples and presumably tissues in levels that were below our level of detectability (approximately 10 ppb). Concentrations of these compounds had to be estimated from knowing the concentration of the compound in solubilizer samples and the dilution necessary to produce 100 ppb in tank concentrations. This method is questionable because we could not accurately assess losses of components through volatilization, ionization, or adsorption at the tank level. Recent preliminary testing of a photoionization detector on our gas chromatograph indicated that all monocyclic components could be detected in extracted water samples from exposure tanks. This increased sensitivity is about 100-1000 times better than attained using a conventional flame ionization detector. Water and tissue samples will be retested through the new detector and results will be included in the final report.

MANUSCRIPTS IN PREPARATION

Benville, Pete E. and D. Ross Smart. Gas chromatography techniques for measurement of monoaromatics using a photoionization detector.

Benville, Pete E., Thomas G. Yocom, and Jeffrey M. O'Neill. Simple, continuous-flow systems for dissolving the water-soluble components of crude oil into seawater for acute or chronic exposure of marine organisms.

Smart, D. Ross, Thomas G. Yocom, and Jeannette A. Whipple. Comparative uptake and retention of the water-soluble fraction of Cook Inlet crude oil by clams, Tapes semidecussata and Protothaca staminea.

Whipple, Jeannette A. and Maxwell B. Eldridge. Review paper on the effects of monoaromatics on fish.

Whipple, Jeannette A., Thomas G. Yocom, D. Ross Smat, and Meryl H. Cohen. Effects of low-level hydrocarbon pollution on spawning marine organisms.

Yocom, T. G., D. R. Smart, M. H. Cohen, P. E. Benville, and J. A. Whipple. Uptake and retention of water-soluble components of crude oil from good (Tapes semidecussata) and water by starry flounder (Platichthys stellatus).

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5. BIOAVAILABILITY OF HYDROCARBONS AND HEAVY METALS TO MARIN DETRITIVORES FROM OIL-IMPACTED SEDIMENTS (RU 454)

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INTRODUCTION

The objectives of this research program may be separated into three subject areas: (1) To determine the bioavailability of sediment-sorbed petroleum hydrocarbons to detritivores, (2) To determine the effect of oil on bioavailability of trace metals and (3) To determine the effects of oiled sediment on detritivores. In the first year of this study, primary emphasis was placed on objectives 1 and 2, but some observations of organismic condition were made as part of uptake experiments. By using both field and laboratory experiments, organisms were examined under both controlled and natural conditions. Hydrocarbon uptake experiments utilized ultraviolet spectrophotometry, gas chromatography, and liquid scintillation count of C^{14}-labelled compounds. Findings regarding bioavailability of hydrocarbons were therefore verified by using different techniques to measure the uptake of a range of petroleum hydrocarbons. X-ray fluorescence was used to analyze sediment and tissue samples for a wide range of trace metals.

These studies were conducted to assess the potential damage of oil-contaminated marine sediments on benthic organisms and the availability of this material to marine food-webs and eventually man. It is necessary and important to understand the interactions (transport) which occur when oil reaches sediments in either intertidal or subtidal marine ecosystems. The relevance of our research findings to these major questions will be addressed in the conclusion section after a summary of each research topic has been presented. Since the majority of our research has been submitted for publication, no detailed methods section is included and the results presented represent an overview.

DISCUSSION

UPTAKE OF HYDROCARBONS FROM SEDIMENT

The results of both our field and laboratory experiments were consistent with previous findings that naphthalenes in oil-contaminated sediments are not readily accumulated by benthic detritivores (Rossi, 1977; Anderson et al., 1977; Roesijadi et al., 1978a) and extended the data to include aliphatic and higher molecular weight aromatic compounds.

In the field experiment described in this report (Table 1) uptake of aliphatic and diaromatic petroleum hydrocarbon concentration was examined in 3 benthic animals exposed to sediment contaminated with Prudhoe Bay crude oil. The concentrations of hydrocarbons in sediment (887 μg/g initially and 420 μg/g after 60 days) were extremely high, but consistent with levels reported for heavily contaminated areas (Gilfillan, 1976; Krebs and Burns, 1977). In contrast, tissue hydrocarbon levels reported here after 40 and 60 days of exposure were relatively low. Maximum levels observed in tissue did not exceed 6 μg/g combined aliphatic and diaromatic hydrocarbons. The concentration of C_{12}-C_{28} n-alkanes in tissue was roughly equivalent to those of the diaromatic hydrocarbons, with the dimethylnaphthalenes predominant in the latter fraction. Feeding type of test species may have influenced the bioavailability of hydrocarbons. The deposit-feeders accumulated hydrocarbons to a greater extent than the suspension-feeder; this pattern was evident at 40 days. At 60 days, however, the data seemed to indicate a difference in uptake patterns between the sipunculids and the bivalve species, and suggested intrinsic differences in abilities of different species to accumulate hydrocarbons. Hydrocarbon concentrations in the sipunculid Phascolosoma agassizii at this time were not different from 40 days levels; whereas, those in the bivalve Macoma inquinata and Protothaca staminea, represented measurable increases.

Suspension-feeders would not ordinarily be exposed directly to contaminated sediments or interstitial water. Dilution of leached hydrocarbons by overlying seawater can probably explain the lower levels observed in Protothaca staminea. When compared to other organisms, bivalves have been shown to take up hydrocarbons from seawater at a slow rate for a prolonged period of time until equilibrium is reached (Stegeman and Teal, 1973; Neff et al., 1976). Tissue hydrocarbon concentrations as high as several hundred μg/g were reported for oysters and clams. Thus, it appears that neither M. inquinata and Protothaca staminea approached their maximum capacity to accumulate hydrocarbons during our study. With the sipunculid Phascolosoma agassizii, however, tissue levels of naphthalenes were stable after the first 40 hr of a 2-week exposure for oil-contaminated sediment (Anderson et al., 1977). Those results were consistent with our observations in longer exposures.

When exposed to detritus containing radiolabelled polycyclic aromatic hydrocarbons spilled into Prudhoe Bay crude oil, accumulation of these compounds by M. inquinata was also low. We found that compounds directly associated with particulate matter were less available for uptake than that fraction released to the surrounding seawater (Table 2). Concentration factors for uptake from sediment and uptake from seawater supported such conclusions. Based on our findings, we suspect that hydrocarbons present in interstitial water may be a prime source of

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Table 1. Aliphatic and diaromatic hydrocarbons in samples[1] of *Phascolosoma agassizii*, *Macoma inquinata*, and *Protothaca staminea* exposed to oil-contaminated sediment. From Roesijadi *et al.* (1978b).

Species	Treatment	Hydrocarbon Concentrations (μg/g wet weight) Saturates C_{12}-C_{28}	N	MN	DMN	Total[2] Diaromatics	Total Hydrocarbons measured
P. agassizii	Control	<0.10	<0.005	<0.005	<0.01	<0.02	<0.12
M. inquinata		<0.10	<0.005	<0.005	<0.01	<0.02	<0.12
P. staminea		<0.10	<0.005	<0.005	<0.01	<0.02	<0.12
P. agassizii	40 day exposed	1.90	<0.005	0.23	0.60	0.83	2.73
P. agassizii		0.73	<0.005	0.01	0.15	0.16	0.89
M. inquinata		0.69	<0.005	0.06	0.89	0.96	1.65
P. staminea		<0.10	<0.005	<0.005	<0.10	<0.02	<0.12
P. agassizzi	60 day exposed	1.48	0.01	0.06	0.18	0.25	1.73
M. inquinata		0.54	0.02	0.27	2.39	2.68	3.22
M. inquinata		3.62	0.02	0.26	1.96	2.24	5.86
P. staminea		0.10	<0.005	0.02	0.16	0.18	0.28
M. inquinata	60 day exposed	0.35	<0.005	0.03	0.96	0.99	1.34
	7 day depurated	0.15	<0.005	0.02	0.20	0.22	0.37
P. staminea		0.03	<0.005	<0.005	<0.01	<0.02	0.03
		0.02	<0.005	<0.005	0.01	0.01	0.03

[1] Each sample consisted of 2 to 4 pooled individuals; clams were shucked prior to extraction.

[2] Total diaromatics include naphthalene (N), methylnaphthalenes (MN), and dimethylnaphthalenes (DMN).

Table 2. Uptake of ^{14}C-polyaromatic hydrocarbons from sediment by *Macoma inquinata*. Clams were exposed to sediment containing 2000 ppm crude oil spiked with 10 μCi of the hydrocarbon indicated in the table. From Roesijadi *et al.* (1978b).

Parameter	2-Methyl-Naphthalene	Phenanthrene	Chrysene	Dimethyl-Benzanthracene	Benzo(a)pyrene
Net Uptake From Sediment[1] *(μg/g)*	0	0.096	0.308	0.297	0.059
Uptake From Seawater *(μg/g)*	0.048	0.038	0.297	0.856	0.037
Sediment Magnification Factor[2]	0	0.056	0.029	0.039	0.057
Seawater Magnification Factor[3]	3.2	5.89	105	295	420

[1] Calculated As Indicated in Text

[2] Sediment Magnification Factor = Net Uptake/Geometric Mean Concentration In Sediment

[3] Seawater Magnification Factor = Uptake From Seawater/Geometric Mean Concentration In Seawater.

contamination for infaunal species and probably accounted for much of the uptake by Phascolosoma agassizii and M. inquinata in the field experiment described above. Concentration factors for 7 da exposures (based on uptake from seawater) correlated positively with the increasing molecular weights of the aromatic hydrocarbons examined here. Such a relationship was not unexpected and predictable from our current understanding of bioconcentration of organic compounds and partition coefficients (Neely et al., 1974). During continuous exposure to aromatic hydrocarbons, concentrations in tissue would be expected to reach an equilibrium with external concentrations. Lighter molecular weight hydrocarbons would tend to approach equilibrium values at a faster rate than heavier compounds. Actual equilibrium concentrations in tissue would depend also on the concentration in the external medium. Our longer-term tests indicated that phenanthrene concentrations reached equilibrium, presumably with molecules leached from sediment to seawater, within 2 da and chrysene within 14 da (Figure 1). Subsequent declines in tissue hydrocarbon concentrations reflected new equilibrium concentrations in response to declining levels in sediment. Benzo(a)pyrene, however, appeared not to have reached an equilibrium during our experiment since levels in tissue increased throughout the entire exposure period despite the declining levels in sediment.

BIOAVAILABILITY OF TRACE ELEMENTS FROM OIL-CONTAMINATED SEDIMENT

Numerous trace elements including heavy metals occur in crude oil at low concentrations (Shaw et al., 1970a, 1970b; Hitchon et al., 1975). Little is known about the dynamics of petroleum-derived heavy metals in the marine environment. Accumulation of specific heavy metals from sediment by benthic organisms has been described in numerous studies (Byran and Humerstone, 1971, 1973a, 1973b; Renfro, 1973; Hess et al., 1975, Luoma and Jenne, 1975; Renfro and Benayoun, 1975). The results, however, may not be directly applicable to petroleum-sediment-organism interactions since organic coatings on the surface of sediment particles can inhibit metals uptake (Luoma and Jenne, 1975). The presence of petroleum may interfere with the "normal" uptake kinetics of heavy metals from sediment. The low concentrations of trace metals in crude oil (e.g., 14 μg/g in Prudhoe Bay Crude oil) suggest that the contribution of those metals to the environment will be very low. For example, sediment contaminated with 2,000 μg/g Prudhoe Bay Crude will contain approximately 0.048 μg total trace metals contributed by the oil, assuming that all metals in oil remained in the sediment. Compared to the normal levels of trace metals in marine sediments, that from oil would be negligible. Feder et al. (1976) were not able to detect any changes in sediment metals content as a result of contamination by Prudhoe Bay crude oil. However, experimental examination of the possible effects of oil on the uptake of trace metals is lacking. Bioavailability of petroleum-derived trace metals from oil-contaminated sediment or the effect of petroleum hydrocarbons on routine uptake of trace metals have not

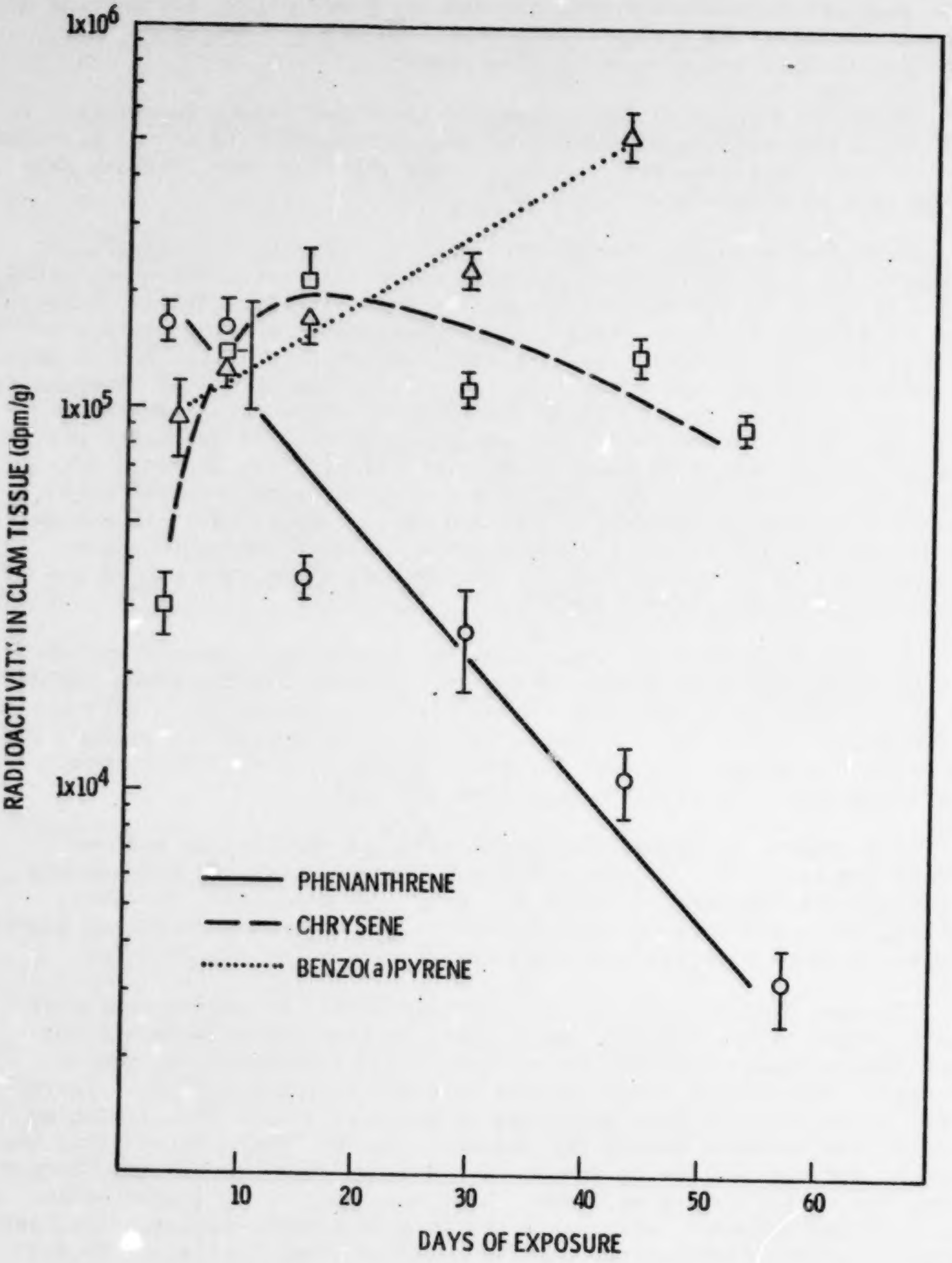

Figure 1. Radioactivity in clam tissue at intervals during exposure to HC-phenanthrene, -chrysene, or -benzo(a)pyrene.

been reported. We have been investigating the uptake of trace metals from sediment contaminated with Prudhoe Bay Crude oil in two species of deposit-feeders: Phascolosoma agassizii and Macoma inquinata. Our findings to date are present in this report.

Sipunculids Phascolosoma agassizii and clams Macoma inquinata were collected from intertidal regions of Sequim Bay and held at the Battelle Marine Research Laboratory. Holding tanks contained raw, flowing seawater of approximately 10°C and $30^{o}/oo$.

Experiments were of two general designs. First, individuals of Phascolosoma agassizii were exposed to oil-contaminated sediment (~2000 µg/g) prepared by mixing sand and oil (Anderson et al., 1977). Exposures were conducted in sediment trays immersed in holding tanks containing flowing seawater. Animals were sampled after 1, 7, and 14 days of exposure and 14 days depuration. Whole animals or tissue homogenates were dried and analyzed by x-ray florescence. Secondly, clams Macoma inquinata were exposed to oil-contaminated detritus as described previously for experiments on short-term uptake of aromatic hydrocarbons. Exposure lasted for 2 weeks. Fifteen g of oil-contaminated detritus (2000 µg/g) were supplied initially and on the seventh days of exposure. Clams were sampled at the end of exposure, allowed to depurate gut contents for 24 h, then dried. Tissue from 10 clams were pooled and analyzed for x-ray florescence.

A separate experiment was conducted to determine natural variability of trace metals in Macoma inquinata. Immediately following collection, 100 clams were placed in clean, flowing seawater for 5 days to allow depuration of gut contents. Following the depuration period, the clams were dried and analyzed for trace metals by x-ray fluorescence. Ten samples of 9 to 10 clams each were analyzed.

Two samples of Prudhoe Bay Crude oil were analyzed by neutron activation analysis. Concentrations of trace elements in Prudhoe Bay Crude oil are presented in Table 3. Only four compounds, vanadium, cobalt, zinc, and bromine, were present at levels above detection limits. The total trace elements concentration was approximately 24 µg/g.

Several difficulties arose in the experiment to assess uptake of trace metals by Phascolosoma agassizii. Primary among these was the fact that exposure sediment became anaerobic in the early stages of exposure, and exposed worms crawled onto the sediment surface. Therefore, worms may have been releasing to seawater rather than taking up metals from sediment during the exposure period. Such observations may account for the decreasing levels of compounds such as chromium, manganese, and nickel during exposure. Most were present at levels below those of the sediment, although a few such as nickel, copper, zinc, and manganese, were elevated compared to sediment concentrations. No discernable differences were observed between control and exposed groups. Values for aluminum, silicon, and titanium can be attributed to metals associated with sediment in the gut (Table 4).

able 3. Trace element concentrations in Prudhoe Bay Crude oil Samples represent oil from two different barrels and were analyzed by neutron activation analysis.

Element	Concentration (µg/g)	
	Sample 1	Sample 2
Na	<0.06	0.097
Mg	<30	<33
Al	<0.5	<0.5
Cl	<1	0.95
K	<4	<1.4
Sc	<0.001	<0.001
V	20.9	18.0
Cr	<0.21	<0.15
Mn	<0.04	<0.02
Fe	<1.6	<1.7
Co	0.018	0.017
Cu	<5	<3
Zn	0.31	0.31
As	<0.03	<0.01
Se	--	<0.3
Br	5.73	2.75
Rb	<0.06	<0.08
In	<0.005	<0.003
Sb	<0.002	<0.002
Cs	<0.002	<0.001
Ba	<23	<8
La	<0.01	<0.01
Sm	<0.002	<0.001
Eu	<0.001	<0.001
Tb	<0.007	<0.006
Ta	<0.04	--
Hg	<0.03	<0.03
Th	<0.008	<0.006

Table 4.

Treatment	Concentration (µg/g dry weight)									
	Al	Si	P	S	Cl	K	Ca	Ti	V	C
Control										
sediment	60,500	35,500	-	-	-	8,200	29,400	4,550	33	69
sediment	60,700	37,200	-	-	-	8,500	30,100	4,810	61	703
animals from field	7,600	3,150	1,905	8,710	43,490	6,706	6,208	204	7.8	42
animals held in lab in sediment	5,070	2,716	1,606	9,953	38,650	7,600	2,344	242	6.0	29
animals held in lab 2 days out of sediment	5,800	1,310	395	10,500	49,700	6,820	3,480	153	<6	45
Exposed										
1 day - whole animals	5,000	437	1,536	11,300	43,400	7,350	1,850	100	<5	14
1 day - homogenate	3,400	1,300	790	9,490	32,034	6,230	2,326	145	<6	142
1 day - homogenate	2,000	1,224	1,680	9,180	40,633	7,100	2,684	210	9.8	94
7 days - homogenate	19,450	1,584	2,408	11,140	46,190	9,174	3,986	128	6	47
14 days - homogenate	12,630	1,636	1,216	11,150	49,320	9,478	2,323	83	<4	29
14 days + 14 days depuration - homogenate	20,990	1,673	<727	13,300	92,050	10,230	3,112	58	<4	15

	Mn	Fe	Ni	Cu	Zn	Se	Pb	As	Br
Control									
sediment	443	17,900	<30	<2	<3	14	5.5	1.5	3.9
sediment	442	17,700	<30	<2	<3	12	2.0	0.8	3.5
animals from field	1,120	4,647	20	18	132	3.4	6.4	9.4	837
animals held in lab in sediment	452	4,256	24	9.2	109	8.0	5.9	12	751
animals held in lab 2 days out of sediment	1,316	3,994	22	15	158	3.8	9.3	10	920
Exposed									
1 day - whole animals	180	2,824	19	20	154	3.1	5.5	10.5	822
1 day - homogenate	1,087	3,673	49	21	251	4.5	15.5	13.1	1,026
1 day - homogenate	1,385	3,947	44	19	185	4.2	19	12	814
7 days - homogenate	598	3,494	31	12	145	<.3	9	12	712
14 days - homogenate	273	2,629	13	12	80	<.5	<1	12	696
14 days + 14 days depuration - homogenate	123	2,241	9	13	112	<.5	<1	11	810

Levels of trace metals in *Macoma inquinata* (Table 5) were generally similar to those of *Phascolosoma agassizii* with few exceptions: concentrations of chromium, manganese, iron, and nickel were substantially lower in *M. inquinata* and occurred despite the fact that metals concentrations in the respective sediment of the two species showed considerable variations. Nickel, zinc, and selenium were magnified in clam tissue, compared to sediment levels. Oil exposure did not affect metals concentrations in *M. inquinata*. With the exception of titanium and lead, individual variability was relatively low for all metals (Table 6) (combined coefficient of variation = 12.3 ± 5.5 S.D.). Titanium was most likely to be associated with sediment remaining in the gut and would therefore be more likely to exhibit a degree of inconsistency. Concentrations of lead approached detection limits of the technique. Only three samples possessed measurable quantities of lead. Only values for chromium and manganese in the previous experiment did not fall within the 95% confidence limits determined in this study.

Our results, to date, suggest that petroleum hydrocarbons have little effect on trace metals concentrations of *Phascolosoma agassizii* or *Macoma inquinata*. However, additional work is obviously required before more definitive statements can be made. The use of neutron-activated oil and sediment samples will aid in our future studies on this subject.

CONDITION INDEX AND FREE AMINO ACIDS OF *MACOMA INQUINATA* EXPOSED TO OIL-CONTAMINATED SEDIMENTS

Our study has shown that both condition index and certain free amino acids in *Macoma inquinata* were significantly altered by exposure to oil-contaminated sediments (Tables 7,8). Adequate sample size was an important factor in demonstrating statistically significant reductions in condition. The reduction in condition index in exposed clams, although small (~10%), provided evidence of a deterioration in nutritional state. A decrease in bivalve condition index is an indication that affected clams may have been in a state of negative-energy balance; in other words, metabolized energy exceeded energy consumed as food. Utilization of endogenous storage products such as tissue protein, lipid, and carbohydrates may have been necessary to provide the balance of the energy for metabolism under such conditions (Gabbott, 1976). Condition index of oysters and mussels have been closely correlated with tissue glycogen content (Walne, 1970; Gabbott and Stephenson, 1974; Gabbott and Bayne, 1973).

Using the criteria proposed by de Wilde (1975) for *Macoma balthica*, a value of > 10 represents good condition, ~8 moderate, and < 6 poor. If these criteria are applicable to *M. inquinata*, the clams in this study possessed mean values for condition index which ranged from good to moderate. Condition in bivalves is known to undergo seasonal variations which are reflective of reproductive and nutritional state (Walne,

TABLE 5. Uptake of trace elements by *Macoma inquinata* exposed to oil-contaminated detritus (~2000 µg/g total hydrocarbons).

Treatment	Cl	K	Ca	Ti	V	Cr	M
	- - - - Concentration (µg/g dry weight) - - -						
Control							
Initial sed.	16,800	11,900	21,100	4,690	80	145	668
2-wk sed.	16,400	11,600	20,900	4,610	109	101	706
Initial tissue	52,900	13,000	1,970	21	<4	<2	11
2-wk tissue	55,200	13,900	1,590	29	<4	2.7	12
2-wk tissue	51,100	12,500	2,030	55	<4	5.6	19
Exposed							
Initial sed.	15,400	11,100	19,600	4,460	84	126	682
2-wk sed.	16,600	11,400	20,400	4,500	73	97	706
2-wk tissue	55,500	13,200	1,870	42	<4	5.5	14

Treatment	Fe	Ni	Cu	Zn	Se	Pb	As
Control							
Initial sed.	38,800	<36	31	85	1.3	10	9.
2-wk sed.	38,600	<32	34	92	1.4	12	13
Initial tissue	284	4.2	7.7	199	3.1	<1.2	11
2-wk tissue	370	3.5	9.1	210	3.1	<1.3	11
2-wk tissue	516	4.9	10.3	202	2.7	<1.3	11
Exposed							
Initial sed.	37,400	<43	31	84	1.5	10	10
2-wk sed.	38,100	<33	35	90	1.3	13	10
2-wk tissue	535	3.3	7.5	163	2.9	2.1	8

Table 6. Analysis of trace elements in *Macoma inquinata* by x-ray fluorescence. Estimation of sample variability.

Element	Sample size[1]	Concentration (µg/g) $\bar{X} \pm 2$ S.E.
P	10	4,651 ± 686
S	10	15,374 ± 591
Cl	10	53,859 ± 3,695
K	10	13,504 ± 245
Ca	10	2,003 ± 140
Ti	10	23.7 ± 9.5
V	3	3.58 ± 0.45
Cr	5	3.92 ± 0.60
Mn	10	9.136 ± 1.043
Fe	10	315.2 ± 31.3
Co	4	2.497 ± 0.442
Ni	10	3.282 ± 0.391
Cu	10	8.108 ± 0.374
Zn	10	195.2 ± 12.5
Ga	10	n.d.[2]
Hg	10	n.d
Se	10	3.177 ± 0.188
Pb	3	0.815 ± 0.680
As	10	10.319 ± 0.368
Br	10	262.5 ± 17.8
Rb	10	n.d.
Sr	10	29.59 ± 2.46

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[1] In sample size <10, the remaining samples (= 10-n) were below detection limits. Nine to ten clams comprised a single sample.

[2] n.d. = not detectable.

Table 7. Condition index of *Macoma inquinata* exposed to oil-contaminated sediment in the second experiment. Exposure was conducted in the field only. (From Roesijadi and Anderson, 1978).

Treatment	Sample size (n)	Condition index
Control	91	8.92 ± 0.18 (S.E.)***
Exposed	50	7.46 ± 0.28

*** Significant at p<0.001; Student's t test

Table 8. Free amino acid content of *Macoma inquinata* exposed to oil-contaminated sediment in the second experiment. Conducted in the field only. (From Roesijadi and Anderson, 1978).

Amino acid	Concentration (μ moles/g)		
	Control	Exposed	
Alanine	22.53 ± 1.82 (S.E.)	16.80 ± 2.02 (S.E.)	
Arginine	6.89 ± 0.40	4.56 ± 0.73	*
Aspartate	1.44 ± 0.17	0.90 ± 0.27	
Glutamate	2.72 ± 0.22	2.21 ± 0.19	
Glycine	70.25 ± 4.51	43.56 ± 4.99	**
Histidine	0.28 ± 0.03	0.22 ± 0.04	
Isoleucine	0.37 ± 0.02	0.32 ± 0.04	
Leucine	0.63 ± 0.05	0.48 ± 0.05	
Lysine	0.59 ± 0.04	0.41 ± 0.04	**
Methionine	0.20 ± 0.03	0.13 ± 0.02	
Phenylalanine	0.22 ± 0.02	0.19 ± 0.02	
Proline	0.57 ± 0.04	0.74 ± 0.25	
Serine	4.09 ± 0.41	3.24 ± 0.39	
Threonine	1.16 ± 0.05	0.87 ± 0.07	**
Tyrosine	0.37 ± 0.03	0.33 ± 0.03	
Valine	0.54 ± 0.04	0.44 ± 0.16	
Taurine	37.06 ± 1.94	35.08 ± 2.39	
Total	150.57 ± 8.15	110.48 ± 8.24	**
Taurine:Glycine	0.54 ± 0.04	0.89 ± 0.12	*

* Significant at p<0.02, Student's t test

** Significant at p<0.01, Student's t test

1970; Trevallion, 1971; de Wilde, 1975). Periodic sampling of clams from our collection site during the course of this study indicated that condition index of our experimental clams, expecially those used in field exposures, were out of phase with the natural population. In general, clams in this study possessed lower condition index than those collected freshly at the times of experiment termination (13.79 ± 1.63 and 17.01 ± 0.78 for experiments 1 and 2, respectively). Thus, experimental manipulation also was a factor which influenced condition index of clams in this study. The effect of oil-exposure on condition was, therefore, either additive or synergistic with general experimental conditions.

Since arginine, lysine and threonine are considered to be essential amino acids (Mahler and Cordes, 1971), decreases in these substances in oil-exposed clams were also suggestive of alterations in nutritive state. Increased utilization of these amino acids, possibly in protein synthesis, by oil-exposed clams or a decrease in their ingestion with food may have accounted for our observations. The large decrease in glycine content in our oil-exposed clams was consistent with previous studies which examined free amino acid levels in marine animals subjected to pollutant or natural stresses (Jeffries, 1972; Roesijadi et al., 1976; Bayne et al., 1976). As a consequence of the decrease in glycine, the taurine:glycine ration was elevated in oil-exposed calms. The actual values of 0.54 ± 0.06 (S.E.) for control clams and 0.89 ± 0.19 (S.E.) for exposed clams in this study were not directly comparable to those reported by Jeffries (1972) or Bayne et al. (1976) since taurine levels in Macoma inquinata were much lower than those in the bivales Mercenaria mercenaria and Mytilus edulis used in the other studies. Although taurine:glycine ratios may prove useful in identifying bivalves which have experienced stressful environmental conditions, it is evident that the cause of the change in the ratios is due primarily to alteration in glycine content. This pattern has been consistent in the studies conducted to date. Examination of glycine metabolism would certainly be useful in understanding this apparent stress response.

CONCLUSIONS

There appears to be a tissue accumulation pattern associated with the molecular weight of the petroleum hydrocarbon, which is probably dependent on relative partitioning coefficients. From other studies it seems that tissue retention times, while animals are in clean water, increase with the size of the hydrocarbon, and the number of side chains (alkylation). In other words, depuration time increases in the approximate order of: naphthalene > methylnaphthalenes > dimethylnaphthalenes > phenanthrene > methyl-and dimethylphenanthrenes > chrysens > benzo(a)pyrene. No single study with one organism and one type of system has been conducted to produce these data, but the basic pattern appears to exist.

We must assume that tissue retention time for a given compound is independent of the route by which an organism received the contamination. There is some evidence that hydrocarbons entering via food may be retained longer, but there is no reason to believe that there are differences associated with water, interstitial water and sediment routes. Therefore, once the hydrocarbons reach the tissue the rates of release (by various means) should be dependent upon relative solubilities in tissue lipids vs. tissue water (and subsequently surrounding water). The rapid uptake ane short-term retention of naphthalenes we have observed in these and earlier studies are probably explained by uptake from water. In a relatively short time sediments give up (release) naphthalenes, to the interstitial water and this compartment eventually exchanges with the overlying water column. If we assume that this is also the sequence of events for higher molecular weight compounds, then the majority of our findings may be explained by differences in the rates of equilibration between sediment-sorbed and water-born hydrocarbons. This line of thought leads to the conclusion that all uptake observed was via the water (including interstitial water) and ingestion of contaminated particles does not result in significant tissue contamination. This hypothesis would appear to be strengthened by the data on trace metals. These substances were not accumulated by the two test species, even though sediments contaminated with oil were ingested. Either the metals were bound tightly to the particles or there was merely an exchange taking place which resulted in no net uptake.

During both laboratory and field exposures of Macoma to oiled sediment, the condition index of the organisms decreased to a greater extent than occurred in clean substrate. When the sample size was large enough to overcome individual variability, both condition index and free amino acid content of Macoma was shown to be affected by oil contamination. Experimental manipulation alone reduced condition index below that of freshed collected animals, which indicates that a better knowledge of site and seasonal variability must be gained and experimental design should be improved. These parameters, which reflect the energy balance of the species, have been shown to be valid and sensitive measurements for the study of pollutant effects.

The results of various aspects of our research under NOAA/BLM funding through the OCSEAP program have been submitted and accepted for publication by three different publishers (Roesijadi, Woodruff and Anderson, 1978; Roesijadi, Anderson and Blaylock, 1978; Roesijadi and Anderson, 1978). The full references for these manuscripts are listed in the literature cited section and copies of the papers are on file at the OCSEAP offices of Juneau, Alaska and Boulder, Colorado.

The application of information obtained in these studies would appear to be relatively straightforward. It is important to understand the interactions between oil, sediments and benthic organisms, once petroleum has reached the substrate. Present evidence indicates that hydrocarbons bound to sediment particles are not directly available to deposit/detritus-feeding organisms. Leaching from sediment would appear to be controlled by the water solubility of the specific compound, physical-chemical factors in the environment, and microbial activity. The physical energy of the environment would control release rates and the dilution volume available when release occurs will control the extent of uptake by benthic organisms. Even when tissue uptake is relatively low the condition of organisms may be reduced, probably from decreased feeding on contaminated substrate. The recovery of a single generation in oiled substrate or the recovery of a specific benthic habitat, would also be controlled by the physical-chemical and microbiological factors in that environment. Not all of those assumptions are sufficiently validated by scientific investigations, but if future studies are designed to fill the information gaps, we will be in a position to predict the fate and effects of petroleum hydrocarbons in marine sediments.

RECOMMENDATIONS AND FUTURE DIRECTION

There is a need to continue sediment studies to test the hypotheses proposed above regarding the factors controlling sediment recovery after oiling. While generating these data, we can also determine the effects of sediment-sorbed hydrocarbons on infaunal species and the rates and routes of hydrocarbon uptake. Field experiments should be designed to answer a number of questions simultaneously, thus saving time and money. With careful planning, coordination and separate funding a large field experiment in the Alaskan environment could be conducted, using the combined expertise of many OCSEAP investigators to describe the physical, chemical, microbiological and biological parameters associated with the presence of oil on sediments. Such field experiments could either be centered around an application of oil on the intertidal zone or a spill in the nearshore environment.

Variability between types of marine sediments and species of benthic organisms are such that additional data are required to determine the extent of these differences and the controlling factors. We intend to look more closely at fine (mud) sediments and to utilize a deposit feeding polychaete in these studies. It is likely that hydrocarbons will be retained longer in this system and that we will be able to separate hydrocarbon inputs via sediment from those in the interstitial water. Condition index and free amino acids appear to be good indicators of effects on bivalves, but only the latter can be used on soft body animals and it may not be useful. While toxicity from water exposure may be linked to tissue accumulation, effects of oiled substrate may be primarily related to reduced feeding on contaminated detritus and/or sediment. Since highly contaminated substrates are often nontoxic to benthic species in the short-term, we may need to determine the levels of sediment contamination that interfere with normal energy intake over the long-term. While trace metals were not accumulated in our experiments, we will utilize neutron-activated detritus and oil to study exchange rates for metals and to look more closely for uptake.

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6. ACTIVITY-DIRECTED FRACTIONATION OF PETROLEUM

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Our program on activity-directed fractionation of petroleum is in its formative stage so there are no results yet to present and discuss. This presentation ~~will~~ describe the basis for the program, its objectives, and the research plan.

As we are all aware, a great deal of effort has been devoted in recent years to studying the biological effects of petroleum. In addition what might be termed gross smothering effects, as in the oiling of birds and the covering of estuarine plants and animals, many acute and subacute toxic effects of dissolved and dispersed components have been observed at very low levels. The biological studies are frequently supported by chemical analyses in an effort to determine which petroleum components are causing the effects observed.

Generally the chemical analyses are limited to the determination of components that can be detected most easily, the volatile and semivolatile hydrocarbons that can be detected by gas chromatography. These include saturated hydrocarbons such as n-paraffins, isoparaffins, pristane, phytane, and an unresolved envelope of cycloparaffins (naphthenes) and aromatic hydrocarbons such as benzene, naphthalene, biphenyl, phenanthrene, and higher polynuclear aromatic hydrocarbons are of interest because of their mutagenicity or carcinogenicity.

Unfortunately petroleum is also comprised of many other components including nonvolatile and polar components. Although the latter components may represent as much as 40 percent of a fresh crude oil and over 90 percent of a highly weathered oil, they have been largely ignored in biological effects studies and supporting chemical analyses. Our current research program on activity-directed fractionation of crude oil is designed to assess the potential biological activity of these generally ignored petroleum components.

Since normal paraffins are degraded relatively rapidly by microorganisms and the toxic light aromatic hydrocarbons are rapidly lost from an oil slick by dissolution and evaporation, any program concerning the potential long-term biological effects of an oil spill needs to consider the other components remaining in weathered petroleum. Perhaps some of the chemical monitoring programs currently being funded are actually overlooking the most important biologically-active components. It may be that some of the nonvolatile and polar components that currently are not monitored in the OCSEAP program are highly toxic or mutagenic. If so, the monitoring programs should be modified to provide more meaningful data. On the other hand those components may have no significant biological activity at all and we should continue to ignore them. However, we should not ignore them until we have experimental evidence indicating that they are not important. We should not ignore them simply because they are not detectable by the analytical methods usually employed. The objective of our program is to determine whether or not there are important toxic or mutagenic components in fresh or weathered crude oil that are not currently being monitored in the OCSEAP program.

Activity-directed fractionation, as we use the term, applies to an abbreviated and efficient fractionation scheme in which the activity found from biological screening assays of initial fractions is used to direct subfractionation to only those initial fractions that possess activity. The application of this principle to crude oil and the basic design of our research program is shown in Figure 1. The crude oil that we will use in this study include both fresh and weathered Prudhoe Bay crude oil furnished by Auke Bay Fisheries Laboratory. The weathered oil was prepared by exposing fresh oil to a natural marine environment in Auke Bay for two months during the summer. The oil was contained in a bottomless pine board frame attached to a floating dock. It was thus exposed to dissolution effects from below, photochemical oxidation from above, and degradation by microorganisms and other marine biota. It has since been stored in a freezer at -20 C.

Two different types of bioassays will be used, one for mutagenicity and one for toxicity. The mutagenicity assay that will be used is the Ames Salmonella assay in which the ability of a fraction to cause reversions in histidine-deficient bacteria is measured. Ames has shown that the correlation between positive responses in this screening test and carcinogenicity in animal tests is greater than 80 percent. Three different strains of histidine-deficient Salmonella, TA-98, TA-100, and TA-1537, will be used and all tubes will be treated with induced microsomes in order to have the best possible chance of detecting all types of mutagens. Parallel Salmonella toxicity assays will be run and the concentration of each fraction being assayed in the mutangenicity assays will be adjusted if necessary to be below the toxic level.

There are two main problems with the Ames test that need to be considered. First of all it is not very sensitive. Even for a potent mutagen such as benz(a)pyrene, at least 1 μg must be used to give a detectable mutagenic response. Since an interest in mutagenic components such as benz(a)pyrene, B(a)P, in petroleum has already been established by other research, the fractionation scheme must be designed to provide enough of such components to permit their detection in the assay. Secondly, like all bioassays, the Ames test uses an aqueous medium and special methods must be used to incorporate water-insoluble petroleum fractions into the medium. Our initial studies has shown that the addition of the fractions as solutions in tetrahydrofuran (THF) containing a small amount of nonionic dispersant may be satisfactory.

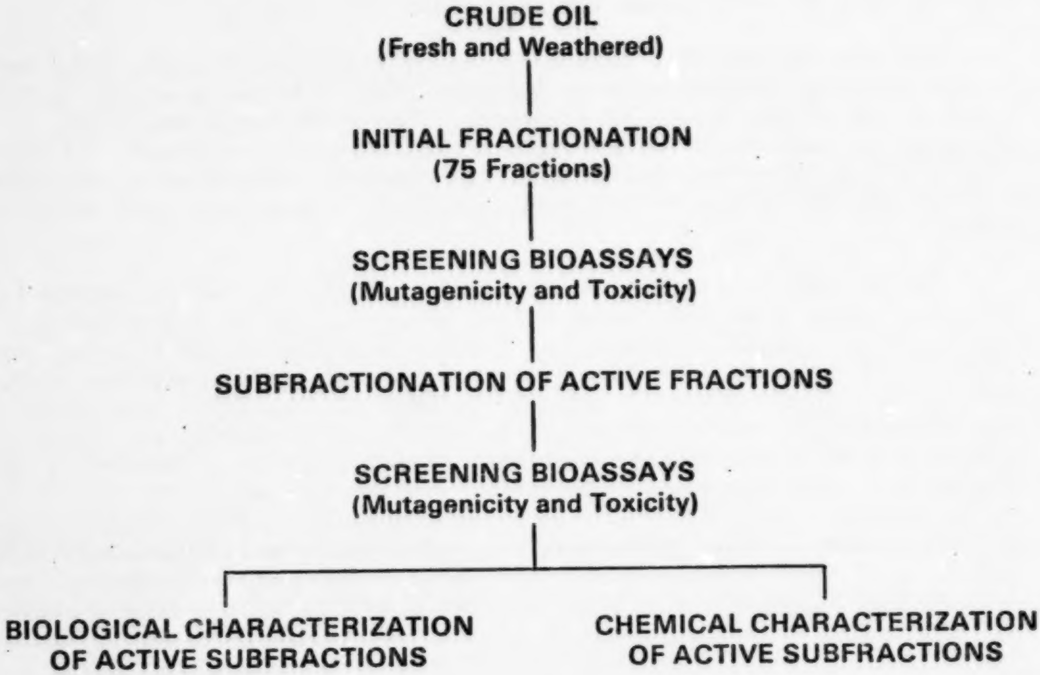
CRUDE OIL
(Fresh and Weathered)
INITIAL FRACTIONATION
(75 Fractions)
SCREENING BIOASSAYS
(Mutagenicity and Toxicity)
SUBFRACTIONATION OF ACTIVE FRACTIONS
SCREENING BIOASSAYS
(Mutagenicity and Toxicity)
BIOLOGICAL CHARACTERIZATION OF ACTIVE SUBFRACTIONS
CHEMICAL CHARACTERIZATION OF ACTIVE SUBFRACTIONS

Figure 1. Basic design for Activity-directed fractionation of crude oil.

The toxicity assay used will probably be an assay utilizing brine shrimp. Initial plans called for the use of an in vitro mammalian cell prescreen confluency assay using BA1B/C 3T3 fibroblasts. This assay is based upon the ability of cells treated with a toxicant to form a confluent monolayer of cells attached to a tissue culture plate. Again the water-insolubility of petroleum fractions is a serious problem. In this case, however, trace amounts of disperants can not be tolerated because they cause detachment of the cells.

Discussions with Dr. Jack Anderson of Battelle's Pacific Northwest Marine Research Laboratory have indicated that a toxicity screening test using brine shrimp should be practical. The assay would be rapid, inexpensive, and would require only small amounts of material. It would also serve as a better indicator of the potential hazard of a petroleum fraction in the marine environment than would a mammalian cell toxicity assay.

The initial fractionation scheme that we plan to use is outlined in Figure 2. An effort will be made to account for all of the starting crude oil in a material balance. The removal of volatiles by a vacuum-stripping process will be the first step to minimize evaporative losses during the determination of residual weights of subsequent fractions. The residue from the vacuum stripping will be treated with petroleum ether or pentane to give the usual separation of pentane insolubles of asphaltenes from the pentane solubles. The pentane solubles will then be fractionated into 17 fractions on the basis of polarity by silica gel column chromatography. The volatiles, the pentane-insolubles, and each of the 17 fractions from the silica gel chromatography will be further fractionated by high pressure liquid chromatography (HPLC) using μ-Styragel columns. This fractionation mode is actually gel permeation chromatography (GPC) which separates components on the basis of molecular size. Since the volatiles fraction will not contain high-molecular-weight components, it will only be separated into three size fractions. All of the other fractions will be separated into four size fractions.

The eluting solvents that will be used to obtain the various fractions from silica gel chromatography are given in Table 1. The saturated and aromatic hydrocarbons will be obtained in the first six fractions. The remaining fractions will yield the carbazoles, benzofurans, and more polar components such as phenols, porphyrins, and carboxylic acids. An initial silica gel fractionation was carried out using acetonitrile instead of THF as the most polar organic eluting solvent since acetonitrile has a considerably higher dielectric constant than THF. However it was found that acetonitrile was a poor eluting solvent simply because it does not dissolve the petroleum fractions very well. Components eluted less rapidly with acetonitrile than with methylene chloride. THF, on the other hand, is a good petroleum solvent and should work much better. A small amount of water and acid will be added to the last eluting THF to completely deactivate the silica gel. The final fraction will be obtained by a 2-day Soxhlet extraction of the silica gel.

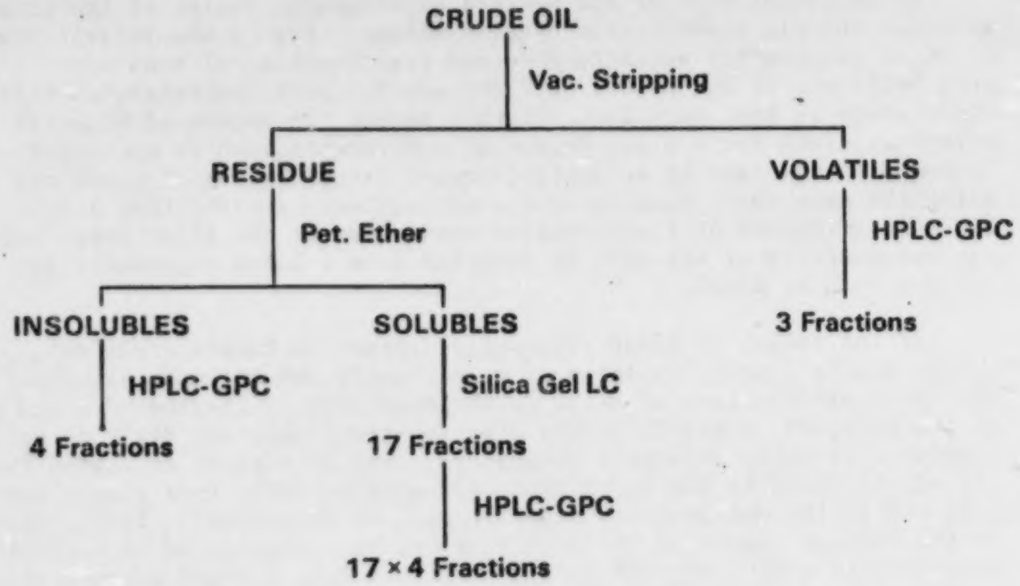

Figure 2. Fractionation methods to be used in this study.

Table 1. Silica Gel Fractionation Scheme.

Fraction No.	Eluting Solvent
1	Petroleum Ether
2	10% CH_2Cl_2 in Pet. Ether
3-6	20% CH_2Cl_2 in Pet. Ether
7-8	40% CH_2Cl_2 in Pet. Ether
9-10	CH_2Cl_2
11-12	20% THF in CH_2Cl_2
13-14	THF
15-16	1% 1N HCl in THF
17	5% H_2O in THF (Soxhlet)

An important part of the overall experimental design of the program involves certain quantitative considerations. From a theoretical standpoint, a program for activity-directed fractionation of a material is most efficient if the number of fractions for each fractionation-bioassay stage is kept very low. In this manner the amount of bioassay effort required for a given degree of subfractionation is minimized. However, in the case of activity-directed fractionation of crude oil using the Ames test, quantitative considerations dictate that a relatively high degree of fractionation must occur at the first stage before any mutagenicity at all will be detected from a known representative mutagen such as B(a)P.

If the amount of B(a)P reportedly present in Kuwait crude oil, approximately 2 μg/g, is taken as a reasonable amount to be expected; if the limit of detection of B(a)P in the Ames test is considered 1 μg; and if the mutagenic activity of the fraction containing the B(a)P is not augmented by other mutagenic components; then it must be concluded that all of the B(a)P in 500 mg of crude oil must be added to a single Ames test before the mutagenicity of B(a)P will be detectable. Furthermore, if the maximum amount of material that can be accommodated by a single Ames test is only 5 mg, and if all fractions from a crude oil fractionation contain equal amounts of material, then it must also be concluded that the crude oil must be fractionated into at least 100 fractions and all of the B(a)P must be in one of those fractions before the mutagenicity of B(a)P will be detectable.

The above quantitative considerations are outlined in Figure 3. Premises 1, 3, and 4 are based on experimental data. Premises 2 and 5 are assumptions which are not expected to be entirely true but nonetheless are useful for calculation purposes for experimental design. The experimental design already described, in which a large number of fractions (75) are obtained at the initial fractionation stage before any bioassays are performed, is based on the above quantitative considerations.

After at least two fractionation-bioassay stages are completed, the second phase of the program will be initiated. The second phase will include the biological and chemical characterization of the most active subfractions. The biological studies will be performed by Dr. Jack Anderson at Battelle's Pacific Northwest Marine Research Laboratory. They will include studies using selected species and life stages of marine organisms such as mysids, benthic amphipods, shrimp, and crab larvae. The chemical studies will be performed at Battelle's Columbia Laboratories and will utilize infrared, ultraviolet, fluorescence, nuclear magnetic resonance, and/or mass spectrometry coupled with derivatization methods and/or degradation methods.

The second phase will very likely require considerably larger amounts of the active fractions of interest. The first phase fractionations will start with 5 to 10 g of crude oil. This may need to be scaled up 10-fold or more for the second phase study.

We are very enthusiastic about this program and expect to have a lot of experimental results to report next year.

PREMISE 1:	**BaP Concentration in Crude Oil = 2 μg/g**
PREMISE 2:	**BaP Is Only Mutagen in Crude Oil**
PREMISE 3:	**Limit of Detection of BaP in Ames Test = 1 μg**
CONCLUSION 1:	**All of the BaP in 500 mg of Crude Oil Must Be Added to Ames Test to Be Detectable**
PREMISE 4:	**Maximum Amount of Sample Accommodated by Ames Test = 50 μl of 10% Solution = 5 mg**
PREMISE 5:	**All Fractions Contain Equal Amounts of Material**
CONCLUSION 2:	**Crude Oil Must Be Fractionated into at Least 100 Fractions and All of the BaP Must Be in One of Those Fractions Before Any Mutagenicity Will Be Detected**

Figure 3. Premises underlying the study design.

7. POTENTIAL INTERACTION OF MICROORGANISMS AND POLLUTANTS FROM PETROLEUM DEVELOPMENT

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When OCSEAP was initiated for the Alaskan continental shelf region in 1975 we proposed to perform three types of microbiological studies. First, a quantitative and qualitative baseline study to describe the existing microbial communities. Second a study of the microbial induced chemical changes in oil composition, i.e., fate of oil. Third, a study on the effects of oil on microbial communities with emphasis on critical ecological mineral cycling processes carried out by microorganisms. The OCSEAP project (RU29/30) that was contracted completely eliminated the third objective due to funding limitations. We were, therefore, unable to initiate any studies on the effects of petroleum hydrocarbons on microorganisms. We were funded to perform studies on baseline reconnaissance of microbial communities and to study the fate of oil in Alaskan continental shelf regions in the Beaufort Sea and the Gulf of Alaska. These studies were initiated in 1975. The Gulf of Alaska studies were later moved to Cook Inlet and Beaufort Sea activities were curtailed during 1976. Figure 1 shows where sampling activities have been carried out. With this as background it is interesting that I have been asked to present data, at this review, on studies of the effects of oil on microorganisms in Alaskan Continental Shelf regions.

Our program has been gathering data on the potential interactions of microorganisms and petroleum. As a result of data gathered ancillary to our reconnaissance and fate of oil studies we have some limited results on the effects of oil on microorganisms, but this is far from the level of scientific data gathering needed for a proper environmental assessment concerning the effects of oil on microorganisms.

Let me describe some of the studies that we have performed in the reconnaissance and fate work and the implications of some of the results of these studies for effect studies.

First, in the reconnaissance studies we have been extensively characterizing microbial communities quantitatively and qualitatively in Beaufort Sea, Gulf of Alaska and in Cook Inlet. Sampling cruises twice a year were used for the reconnaissance studies. We were limited to sampling two times a year because of the massive effort needed for data gathering in microbiological studies following sample collection. Sampling two times a year is probably a justifiable sampling regime in the Beaufort Sea, but in Gulf of Alaska sampling three to four times a

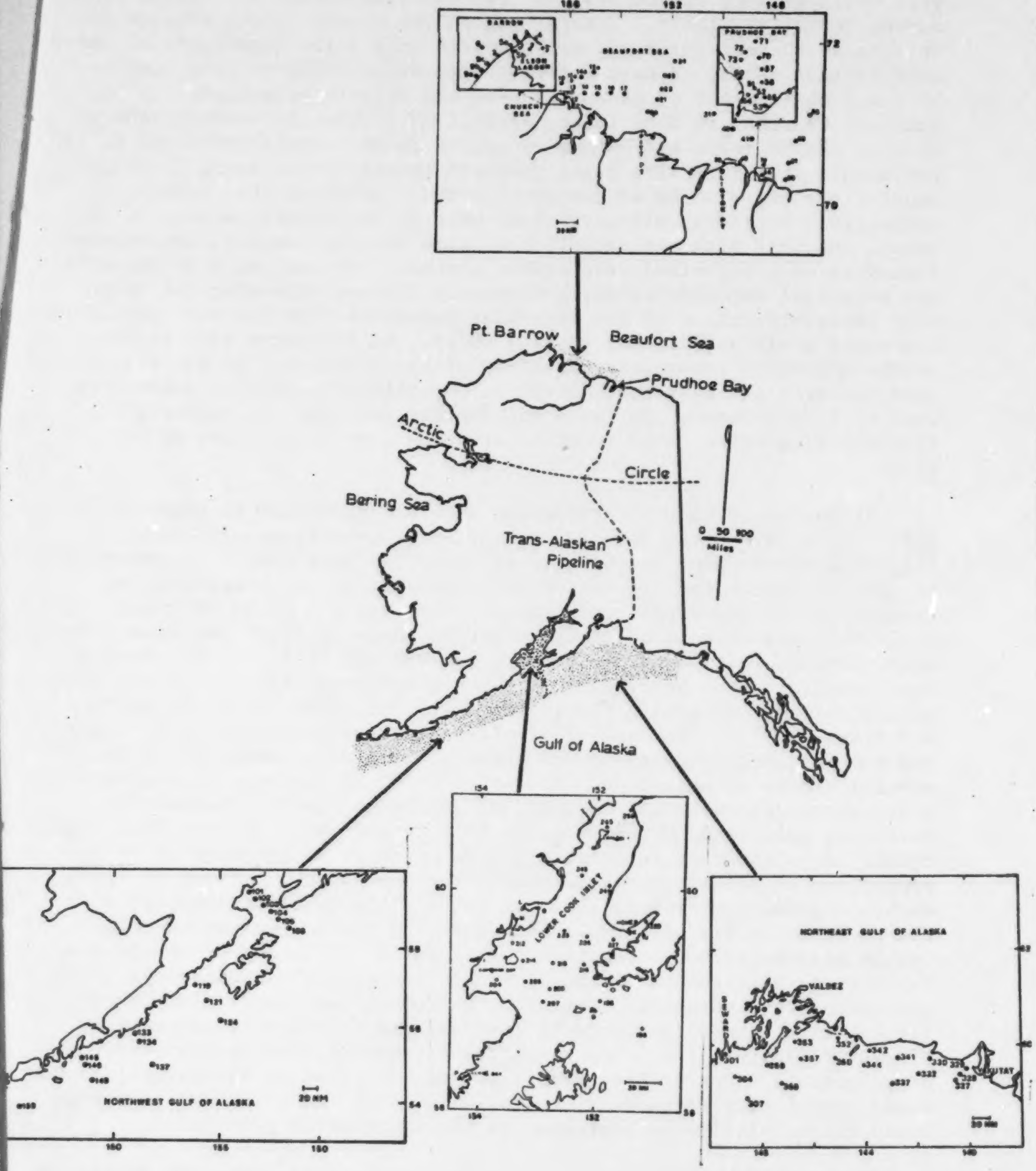

Figure 1. Map of sampling sites in reconnaissance studies.

year would provide better seasonal reconnaissance were sufficient funds and personnel available. I refer you to the annual OCSEAP reports for details of these studies and will mention only a few highlights of these studies that appear to have relevance for considering effects studies. We found that number of microorganisms are surprisingly higher in the Beaufort Sea than in Cook Inlet, or Gulf of Alaska, in surface waters. Many of the bacteria had extensive growth factor requirements and if old pollutants interfere with algal products naturally occurring bacterial populations may also be adversely affected. We found that numbers of hydrocarbon degrading microorganisms were in low concentrations in all areas, but that with the exception of some sea ice samples, hydrocarbon degrading microorganisms were always present. We were able to describe the microbial communities using diversity indices following the extensive characterization of the microbial community that included numerical taxonomic analyses (Kaneko, et al., 1977). We also were able to describe microbial communities in terms of the occurrence of particular features such as physiological types, or ability to utilize substrates such as hydrocarbons. At least for the Beaufort Sea the bacteria are distinct from those found in other seas and show clear signs of adaption.

It was hoped that if sufficient data was generated in these reconnaissance studies that changes in microbial communities, following petroleum development in these areas, could be detected, i.e., we would be able to detect the effects of oil spillages on microorganisms as changes in the microbial communities. What effects would we expect, and would the data base be adequate to detect these effects? We know from other studies that we have conducted for ONR and ERDA that microorganisms respond to the occurrence of petroleum hydrocarbon pollutants both quantitatively and qualitatively (Horowitz and Atlas, 1977a,b; Sextone and Atlas, 1977). The numbers of microorganisms capable of tolerating and metabolizing petroleum hydrocarbon generally increase, often by several orders of magnitude. We would expect an increase in numbers of hydrocarbon degrading microorganisms in response to the presence of polluting petroleum hydrocarbons in Continental Shelf areas. This might result in an increase in total bacterial numbers or an increase in the proportion of the microbial community composed of populations of hydrocarbon degrading microorganisms, or both. This could be detected as a change in the ratio of hydrocarbon degrading microorganisms to total viable microorganisms or as an increase in the frequency of the feature "ability to utilize hydrocarbons" within the microbial community. This enrichment for hydrocarbon degrading microorganisms would also be reflected as a change in diversity (measured with diversity indices). Diversity generally declines significantly in response to the presence of pollutants. Due to the rapid reproductive rates of microorganisms we would expect this response to be rapid and that the microbial community would recover following abatement of the hydrocarbon pollutants.

The data base that we have been developing will allow for detection of such changes in the microbial community. Referring to some related studies performed for the Office of Naval Research on a gasoline spill in a freshwater Arctic lake, we found that immediately after the spillage there was a decline in microbial numbers followed within a few days by increases in microbial numbers to give population numbers higher than originally occurred in the lake (Horowitz and Atlas, 1977a). The microbial community present after the gasoline spillage had a very high proportion of hydrocarbon degrading microoganisms. Indeed, following this spillage the dominant microflora were both capable of hydrocarbon utilization and capable of tolerating lead, since this was a leaded gasoline. We found that one could detect the pattern of spread of gasoline by examining the ratio of hydrocarbon utilizers to total numbers of microorganisms. In this study we also found that the persistence of gasoline pollutants could be detected by monitoring the proportion of the microbial community capable of hydrocarbon utilization. Studying changes in the microbial community was found to be a sensitive method for monitoring the continued effects of this gasoline spillage. We fully expect that similar assessments could be made following marine spillages of petroleum hydrocarbons. Microorganisms do respond to the presence of hydrocarbons with similar increases in population numbers and similar alterations in community structure in Arctic marine ecosystems (Horowitz and Atlas, 1977b). Even chronic low-level input of hydrocarbons can be detected by examining the proportion of microorganisms in the community capable of utilizing hydrocarbons (Atlas and Bartha, 1937; Walker and Colwell, 1976). In some cases the ratio of hydrocarbon utilizing bacteria to total bacteria appears to be more sensitive for detecting chronic oil pollutants than chemical analytical methods that detect the presence of hydrocarbons.

With respect to our fate of petroleum hydrocarbon studies, we have taken two approaches. First a survey approach that paralleled our reconnaissance studies and second, an intensive site study approach. Our reconnaissance has aimed at detecting seasonal and spatial differences in rates of hydrocarbon degradation. These studies indicate that in certain areas, such as the Beaufort Sea, at times of the year such as August through May, rates of hydrocarbon degradation by microorganisms may be very low approaching zero. The persistence of oil pollutants in Alaska Continental Shelf areas will be highly dependent on where and when the contamination occurs.

Our intensive site studies have been designed to allow for detection of biodegradative changes in petroleum composition. Intensive site studies have been conducted to date only for the Beaufort Sea. Some of the studies were initially performed for the Office of Naval Research and are being continued with modifications as part of the OCSEAP program. We have to date recognized three different ecosystems (water, sediment, ice) for intensive study in the Beaufort Sea. For each ecosystem studies were designed to model a likely contamination scenario (Fig. 2):

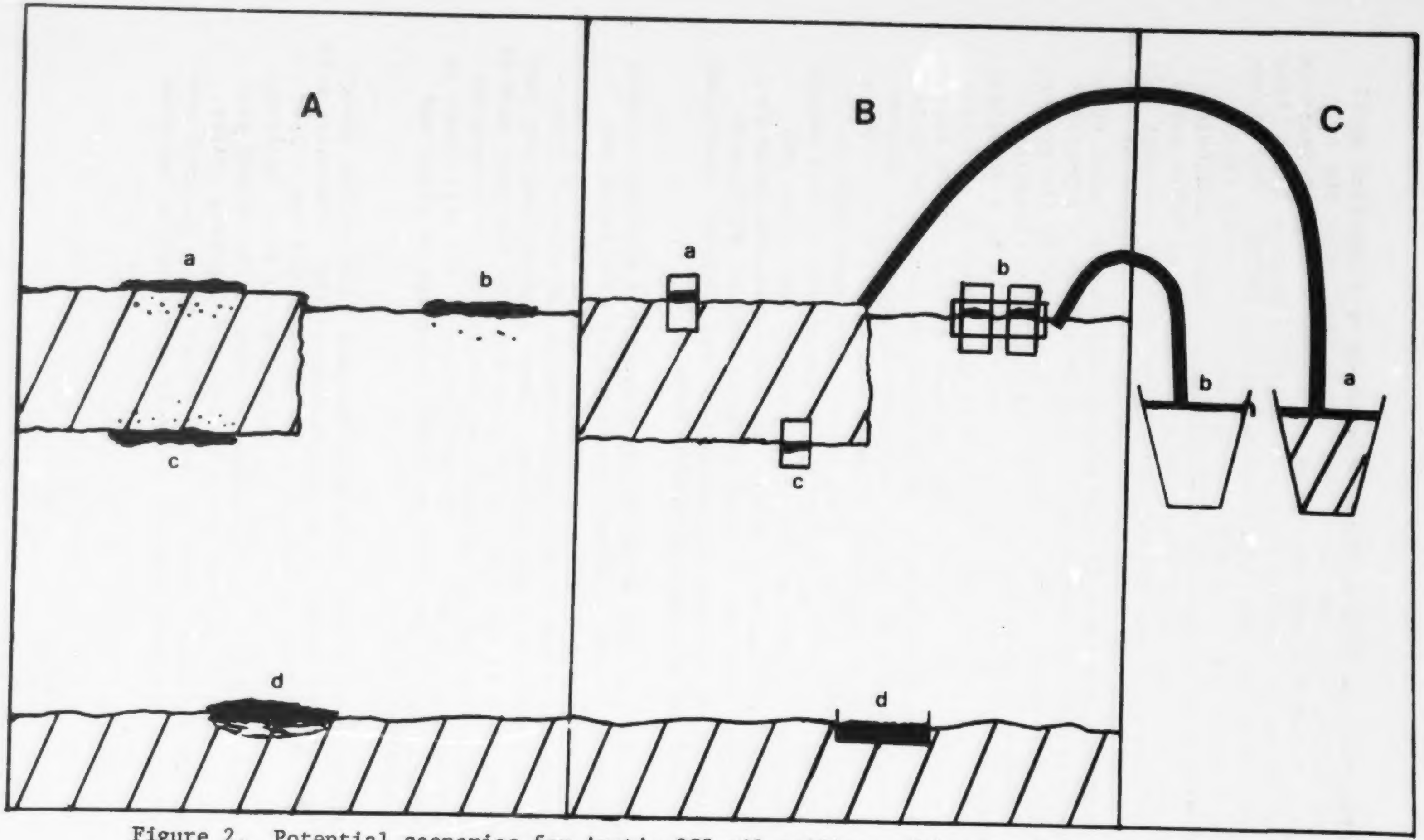

Figure 2. Potential scenarios for Arctic OCS oil spillages (A) and model systems (B,C) for each to study interactions of microorganisms and petroleum hydrocarbons: a) early spring spill over ice; b) summer spill on surface water; c) spring spill under ice; d) spill from buried pipeline, involving bottom sediments. Model experimental systems involve in situ studies (B), as well as studies in containers or microcosms (C).

1) surface water with a scenario of a summer spillage;

2) under ice, with a scenario of an under ice spring spillage;

3) over ice with a scenario of an early spring spill;

4) sediment, with a scenario of breakage or a buried pipeline.

We are currently expanding the scenarios to model mid-winter spills. In each of these ecosystems we have spilled small amounts of oil in a quantitative fashion and periodically recovered the oil for chemical analyses. The oil has been contained but incubated in situ. The analyses performed on the oil typically have been gravimetric determinations of the residual weight of solvent extractable material remaining, gas chromatographic analysis of the saturate fraction to detect changes in the principle components of this fraction, column chromatographic separations and computerized mass spectral determinations for detecting changes in class composition of the various fractions found in petroleum. Table 1 shows some selected results from these studies.

The results are quite different than we would have predicted, based on laboratory and temperate ocean studies. The differences have significance for the design of effects studies, not only on microorganisms but also when dealing with higher organisms. We find that hydrocarbons are degraded very slowly. Persistence from a moderate oil spill must be thought of in terms of years instead of days, or months. Under certain conditions, such as under ice the physical evaporative losses of light hydrocarbons, many of which are highly toxic, are greatly restricted. Similarly, losses of light hydrocarbons in sediment appear to be restricted due to adsorption. We find components, such as naphthalene, still present weeks after spillage under ice. The implication of this finding is that low molecular weight oil components will reside for prolonged periods under certain conditons in Alaska ecosystems. In contrast to sediment and under ice, volatile components are lost within days from open water spillages.

Surprisingly the residual oil recovered from these studies had the same composition of the oil in terms of component classes as the freshly spilled oil (Horowitz and Atlas, 1977b). This is contrary to what one would have expected, since the prediction based on previous studies was that paraffins would be lost before aromatics, leaving behind a residual oil with markedly different class composition than the oil that was spilled. We believe that we can explain our findings in terms of slow degradation rates allowing for co-metabolic changes, a known phenomenon. Oil components would be transformed in rates proportional to their concentration in the oil. The finding does not preclude degradative processes that alter the particular components within a class of compounds. Classes of compounds refers to categories such as chrysene derivatives, naphthalene derivatives, branched paraffins, straight chain paraffins, etc. We do not know whether similar changes will be found if studies were performed in the Bering Sea, Cook Inlet or Gulf of Alaska. Our findings indicate that, at least for the Beaufort Sea, toxicity studies that utilize weathered oil prepared by 48 hour evaporation are probably valid for studying neritic organisms with open water spill scenarios, since the oil remaining in our studies looks very much like weathered oil in terms of chemical class composition. For modelling under ice effects fresh oil should be used.

In the course of our fate of oil studies we have in some cases detected the accumulation of unknown degradation products. We have also sometimes seen the apparent accumulation of synthesized high molecular weight hydrocarbons. We are currently upgrading our analytical capabilities with inhouse GC-MS which will allow us to identify these compounds. Without positive identifications we cannot even speculate as to the potential toxicities of the compounds and the implications for effects work.

As an aside, I would like to comment on chemical analytical methods used for detecting petroleum pollutants. Analysis of petroleum hydrocarbons is necessary both in performing effects studies and as background for such studies. There are a variety of analytical methods available: GC, MS, UV, IR, fluorescence, etc. One method is not "superior" to the others. Each is a powerful analytical method that yields different analytical data. In almost all cases a combination of several analytical methods is needed to accurately describe a petroleum hydrocarbon mixture. One has to recognize chemically what one is detecting and what one cannot detect with each method. One has to ask whether it is appropriate to analyze for specific compounds or for classes of compounds. One must remember that the overall effect of a hydrocarbon mixture can be quite different than the effects of individual components. When considering long term effects one cannot simply analyze for low molecular weight aromatic hydrocarbons, and ignore all else. There are no simple methods for appropriately analyzing petroleum hydrocarbons. There has never been a crude oil that has been completely analyzed. Every crude oil is a chemically unique mixture which may have different effects.

Turning to what studies should be performed to examine the effects of petroleum hydrocarbons on microorganisms, I must emphasize the importance of microorganisms in maintaining ecologic balance and indicate that studies on the effects of oil on microorganisms with respect to metabolic processes are absolutely essential. I fully expect that Dr. Morita will elaborate on this point and bring home to you the importance of such studies in his presentation. I believe that as a model for such studies we can consider a protocol for studying the effects of pesticides on soil microorganisms used for meeting EPA environmental assessment requirements (Atlas, et al., 1978b) as an appropriate framework for OCS studies. Extracting from that protocol experiments would be conducted to determine the effects of oil on:

1) population levels of microorganisms in representative ecosystems,

2) growth rates of representative marine microorganisms,

3) rates of microbial degradation of naturally occurring polymers (e.e. cellulose, chitin, animal protein) in representative ecosystems,

4) rates of microbial transformation of nitrogen compounds (nitrogen fixation, nitrification, denitrification) in representative ecosystems, and

5) rates of microbial transformation of sulfur compounds (sulfur oxidation, sulfate reduction).

Emphasis should be placed on the effects of oil on essential microbial elemental cycling processes. Dr. Morita's group has initiated some studies in this area. We have recently tested methods to examine denitrification processes and, provided funding, could apply these methods to OCS effects studies. Microbiological studies should and have been coordinated between laboratories performing OCS studies. Additional effects studies should consider bioaccumulation of petroleum hydrocarbons and movement of petroleum hydrocarbons through the food web. Microorganisms are integral members of the food web and must be considered with other food web studies.

There is a need for scientifically controlled laboratory microbiological effects studies. There is also a need for *in situ* studies. The *in situ* studies should be coordinated between microbiological groups and with groups from other disciplines. As an example of such multidiscipline studies, in our fate of oil in sediment work, changes in the benthic invertebrate populations were also determined. Those studies showed differential recolonization rates of oiled sediment by benthic invertebrates (Atlas *et al*., 1978a).

In summary there remain three types of microbiological studies which should be included in OCSEAP: reconnaissance, fate, and effects. A series of detailed recommendations to this effect, listing necessary microbiological studies, were prepared for NOAA by a panel of marine microbiologists (NOAA technical memorandum ERL MESA-16). There is a need to extend the reconnaissance into the Kodiak area, Bering and Chukchi Seas, to assess broad spatial and seasonal distribution patterns that would allow for assessment of effects following oil contamination. The fate work should include intensive site studies with different scenarios to model expected contamination incidents. These intensive site studies should be of sufficient size to allow for coordinated fate and effects studies by a variety of disciplines. At some point controlled oil spills or follow-up studies on accidental spills are needed. Microorganisms cannot be ignored in these studies.

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TABLE 1. Chemical analyses of residual oil following 51 days *in situ* exposure on Arctic Ocean surface water during summer 1975.

Column chromatographic analysis of residual oils showing relative percentage of the eluted classes.

	Saturate fraction (%)	Monoaromatic fraction (%)	Diaromatic fraction (%)	Polyaromatic-polar fraction (%)	Polyaromatic-polar fraction II (%)
Fresh crude oil	49.56	16.27	8.83	19.55	5.80
Exposed crude oil	41.65	13.73	8.37	27.60	8.66

NMR spectral analysis

	Hydrogen distribution normalized to 100 total				
	CH_3	CH_2	Alpha-H	Olefinic	Aromatic
Fresh crude oil	30.1	53.1	9.7	0.0	7.1
Exposed crude oil	24.7	56.3	11.3	0.0	7.7

TABLE 1. (continued)

Mass spectral analysis

	Approx concn (%)[a]														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Fresh Crude oil	14.4	11.7	8.3	6.4	4.9	0.3	0.2	8.4	6.2	5.1	8.2	10.2	7.0	6.5	2.0
Exposed crude oil	14.5	11.9	8.2	6.2	4.8	0.3	0.2	8.8	6.0	5.2	8.1	10.5	7.1	6.2	2.0

[a] A, Paraffins; B, monocycloparaffins; C, dicycloparaffins; D, tricycloparaffins; E, tetracycloparaffins; F, pentacycloparaffins; G, hexacycloparaffins; H, alkybenzenes/C_nH_{2n-22} compounds; I, C_nH_{2n-8}/C_nH_{2n-22} aromatics (eg., Tetralin, pyrene); J, $C_nH_{2n-10}/C_nH_{2n-24}$ aromatics (eg., chrysene); K, $C_nH_{2n-12}C_nH_{2n-26}$ aromatics (eg., naphthalene); L, C_nH_{2n-14} aromatics (eg., acenaphthene); M, C_nH_{2n-16} aromatics (eg., fluorene, dihydroanthracene); N, C_nH_{2n-18} aromatics (eg., anthracene, phenanthrene); o, C_nH_{2n-20} aromatics (eg., phenylnaphthalene, ovalene).

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8. MICROBIAL ACTIVITY AND CRUDE OIL-MICROBIAL INTERACTIONS IN WATER AND SEDIMENTS

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OBJECTIVES AND RATIONALE

The role of microbes in the oceans is intimately implicated with the fertility and chemistry of sea water, certain geological processes, food for other forms (because they are so nutritive in terms of proteins, phosphate, liquid, ectocrine compounds, etc.), as well as in the biodegradation of crude oil and man-made compounds.

Due to the above situation the microbiology of the lease areas becomes important not only in relation to the food web but also in the biodegradation of petroleum in the event the latter intrudes accidently into the ecosystem. Also the effect of crude oil on microbial processes must be taken into account.

The data obtained by each microbiological group working in Arctic environments compliments each other so that a more meaningful picture can be obtained concerning the microbiology of the area; which, in turn, provides data that can be used by other groups.

Our research efforts deal mainly with direct counts of the microbial population (epifluorescent counts), heterotrophic activity at in situ temperature, and laboratory studies dealing with microbial functions at low temperature in relation to petroleum degradation (crude oil used from the specific locality), nitrogen fixation, chitinase activity, etc. in the presence and absence of crude oil (recently initiated).

Our main objectives is the study of the following for each lease area:

1. Heterotrophic activity. This is a measure of the relative microbial activity and respiration (mineralization) by the in situ microbial population found in water and sediment samples both from a geographical and temporal viewpoint. Since microbial activity is related to the regeneration of nutrients necessary for phytoplankton growth and production of bacterial biomass, it is important to obtain some measure of the microbial processes taking place. A high rate of activity implies a high growth rate (increase in biomass) of material. Such studies are done within a short period after the time of sample collection (not more than four hours but generally less than two hours)

and determined at a temperature close to the environmental temperature from which the sample was obtained. Areas of high microbial activity may be those in which crude oil undergoes microbial biodegradation the fastest.

2. Direct counts of bacteria. This gives us an idea of the microbial biomass in the water column and sediments. Viable counts do not represent the total number of bacteria present. Direct counts provide us with a clue concerning the microbial biomass as possible food for the higher forms (mainly the meiofauna). Even though counts may not be as high as they could be without the meiofauna, it should be recognized that the bacteria are constantly being cropped by meiofauna and as a result higher counts are not obtained. Such data are important since it is well known that harpacficoid copepods utilize bacterial cells as food and they in turn are eaten by juvenile salmon. Bacteria are also used as food by detritivores, filter feeders, in coprophagy, and they are the main link between POC (particulate organic matter) and DOC (dissolved organic matter). Because of cropping, the data is not meaningful at present (see Future Studies).

3. To evaluate the extent of nitrogen fixation process in sediment as well as in the gut of animals. Nitrogen availability in many instances is a limiting factor in productivity and the enzyme nitrogenase is only found in procaryotic cells. Thus, the fixation of nitrogen by microorganisms is very important in the food web and the impact the intrusion of crude oil has on this process is very important.

4. Crude oil--microorganisms interactions at low temperature. Since one of the main mechanisms to rid an ecosystem of the intrusion of crude oil is microbial biodegradation, studies are being conducted at temperatures close to the temperature found in the areas of study.

Due to the large amount of data obtained from the various field trips (NEGOA, Beaufort, and Lower Cook Inlet) plus laboratory studies and the time allocated for the presentation of our data, we have selected to concentrate our presentation on data collected for Lower Cook Inlet. Data on the other areas has been reported in our quarterly and annual reports.

We have been engaged in reconnaissance studies in Arctic waters and have just been funded (2 months previously) to engage in effect/fate studies.

METHODS

SAMPLING PROCEDURES

Water samples The Niskin (microbiological) sampler was employed to obtain sufficient amount of water aseptically for various analyses.

Water samples are taken from various depths in the water column as well as in different areas. The use of the Niskin water sampler permits the microbiological groups to work with the same sample so that correlations between the two groups as they apply to the Lower Cook Inlet can be made easily (see Fig. 1). In order to avoid sampling errors, all analyses are done on the same water sample.

Sediment samples Sediment samples are taken with a Van Veen grab, Kahl snapper, etc. Again sufficient amount of sample is available for all studies (see Fig. 1).

Benthic organisms Macroorganisms in the various location were collected with an otter trawl or some other standard methods. Upon retrieval of the macroorganism, microbiological samples are taken and immediately frozen for further analysis back in the laboratory at Oregon State University or processed aboard ship for N_2-fixation studies.

HETEROTROPHIC POTENTIAL STUDIES

The techniques used in this study were essentially those of Hobbie and Crawford (Science 159:1463, 1968) as modified by Harrison, Wright and Morita (Appl. Microbiol. 21:698, 1971). When sediment samples were used, a 1.0 ml sample was diluted with a 32 o/oo (w/v) solution of sterile artificial sea water. After dilution, the one ml subsample of the sediment slurry was dried and weighed to determine the dry weight.

All samples were run immediately after collection (generally within 2 hr after collection) at a temperature within 1 C of the in situ temperature from which the sample was obtained.

DIRECT MICROSCOPIC (EPIFLUORESCENT) COUNTS

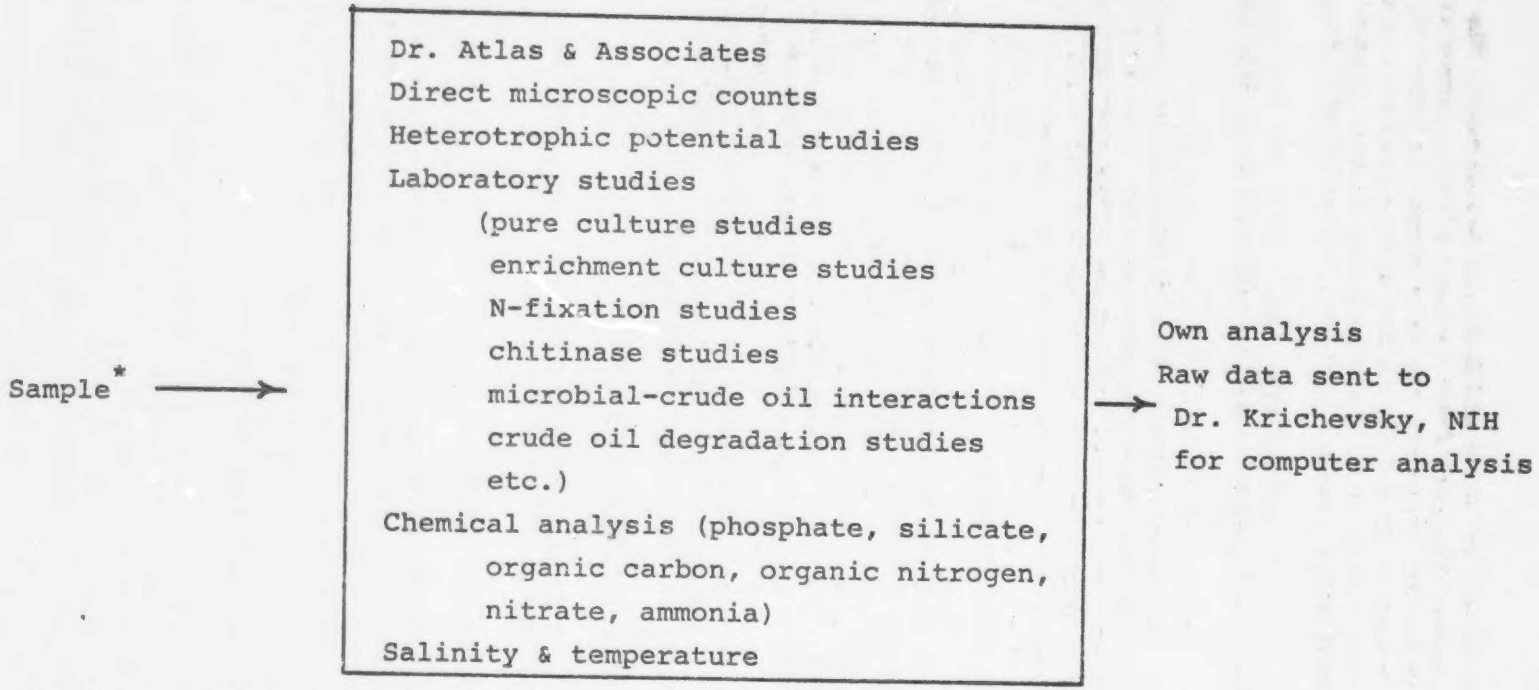
The method of Zimmerman and Meyer-Reil was employed (Kieler Meeresforschungen 30:24, 1974).

DESULFOVIBRIO COUNTS IN SEDIMENT

The minimum dilution method was employed using medium M10E (ZoBell and Morita, Deep Sea Res. 3:6, 1955). The inoculated medium was incubated at 4 C initially when being transported back to the laboratory. Upon arrival the temperature was changed to 8 C and evidence of sulfate reduction was checked after four weeks incubation.

CRUDE OIL DEGRADATION POTENTIAL IN SEDIMENT

These experiments were conducted on sediment samples that were brought back to the laboratory at Oregon State. The samples were kept at or below 4 C between the time they were collected and the start of the experiment. Ten ml subsamples of the sediment were placed into 50 ml serum bottles. To this was added 0.1 ml of Cook Inlet Crude oil


Sample*
Dr. Atlas & Associates
Direct microscopic counts
Heterotrophic potential studies
Laboratory studies
(pure culture studies
enrichment culture studies
N-fixation studies
chitinase studies
microbial-crude oil interactions
crude oil degradation studies
etc.)
Chemical analysis (phosphate, silicate,
organic carbon, organic nitrogen,
nitrate, ammonia)
Salinity & temperature
Own analysis
Raw data sent to
Dr. Krichevsky, NIH
for computer analysis

* Water sample take with Niskin "butterfly" microbiological sampler
Sediment sample taken with Kahl sampler, Van Veen sampler, etc.

Fig. 1. General procedure used in processing samples. Sample size is sufficiently large to handle all the above analyses.

which contained 0.37 μCi pristane and 0.37 μCi hexadecane. The bottles were sealed with rubber stoppers fitted with a plastic bucket containing fluted filter paper. Duplicate subsamples and one acidified control were used to determine crude oil mineralization in each of the sediments analyzed. The samples were incubated at 8 C for 31 days. At the end of the incubation period, the evolved $^{14}CO_2$ was collected and assayed for radioactivity.

NITROGEN FIXATION AND FIXING BACTERIAL CONCENTRATIONS IN SEDIMENTS

Nitrogen fixation in the sediments was determined in the field by using the acetylene reduction method (Stewart et al., Proc. N.A.S.-U.S. 58:2071, 1967). Ten ml subsamples of sediment were added to respective 50 ml serum bottoles: one control and two duplicate samples were used for each analysis. After the bottles were sealed with a rubber stopper, the samples were gassed for one min with He at a flow rate of 10 cc/sec. Ten ml of acetylene was then added to each bottle and the bottles were allowed to incubate for 24 hr before incubation was terminated with one ml of 50% trichloracetic acid (TCA). The controls were treated in the same way before incubation and were used to determine the amount of ethylene that was released abiotically. After the incubation was terminated, the tops of the rubber stoppers were sealed with silicone cement. The bottles were kept at or below 4 C until they could be assayed for ethylene in our laboratory at Oregon State University. The analysis for ethylene was made on a Hewlett Packard model 5830A gas chromatograph. The column used was 1.9 meter of 1/8" stainless steel tubing packed with Porapak R 80-100 mesh and the oven temperature was 40 C. The carrier gas was nitrogen flowing at a rate of 29 cc/min. The resulting levels of ethylene were normalized using incubation times and gram dry weight conversions.

RESULTS AND DISCUSSION

The station locations were presented in Fig. 2. In Fig. 3, 4, and 5 the relative microbial activity as determined by the heterotrophic potential method for the Lower Cook Inlet during the April and October cruises are presented. The highest level of activities were seen in the northern-most stations (Stations 265 and 266) using either glucose or glutamic acid as substrates. Lower values offshore were observed to the southwest of Kamishak Bay, stations 215 and 225 in the center of the inlet, and those found in Kachemak Bay were still lower, and values seen in the south and southeastern sections were the lowest (0.5 to 2.0 μg glutamic acid/liter/hr). This is very similar to the pattern of relative microbial uptake that was seen in water samples analyzed during our fall, 1976 cruise in the same area.

The information from relatively high resolution microbial activity measurements coupled with data on the hydrography and currents of the

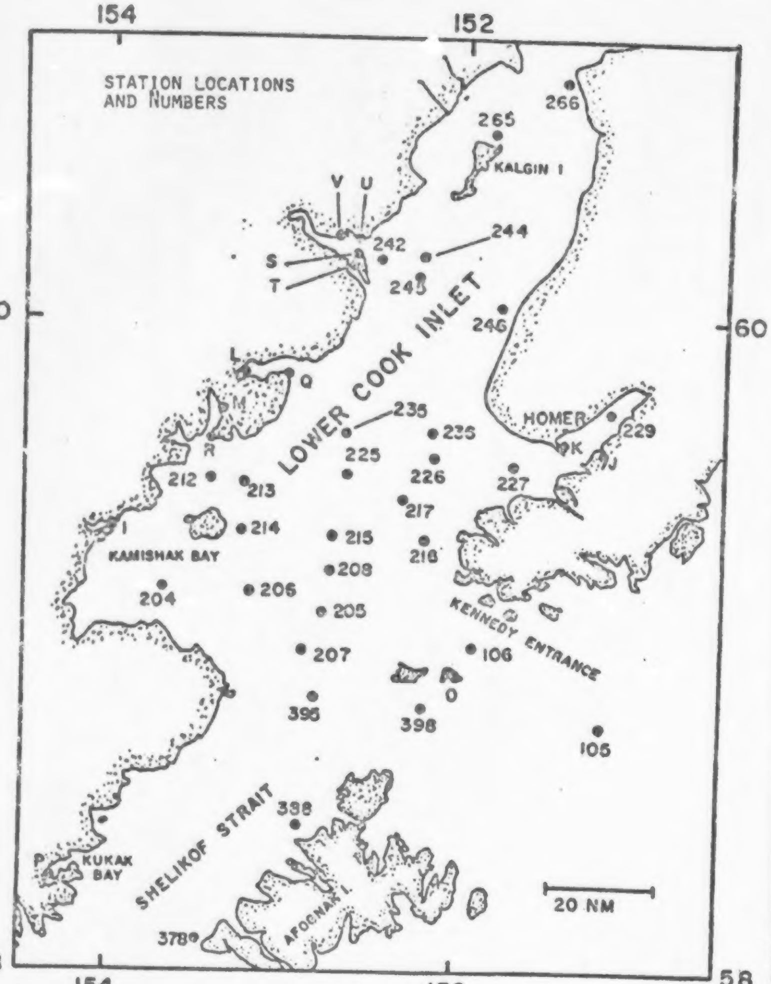


Figure 2. Station locations and numbers for stations occupied in the Cook Inlet area.

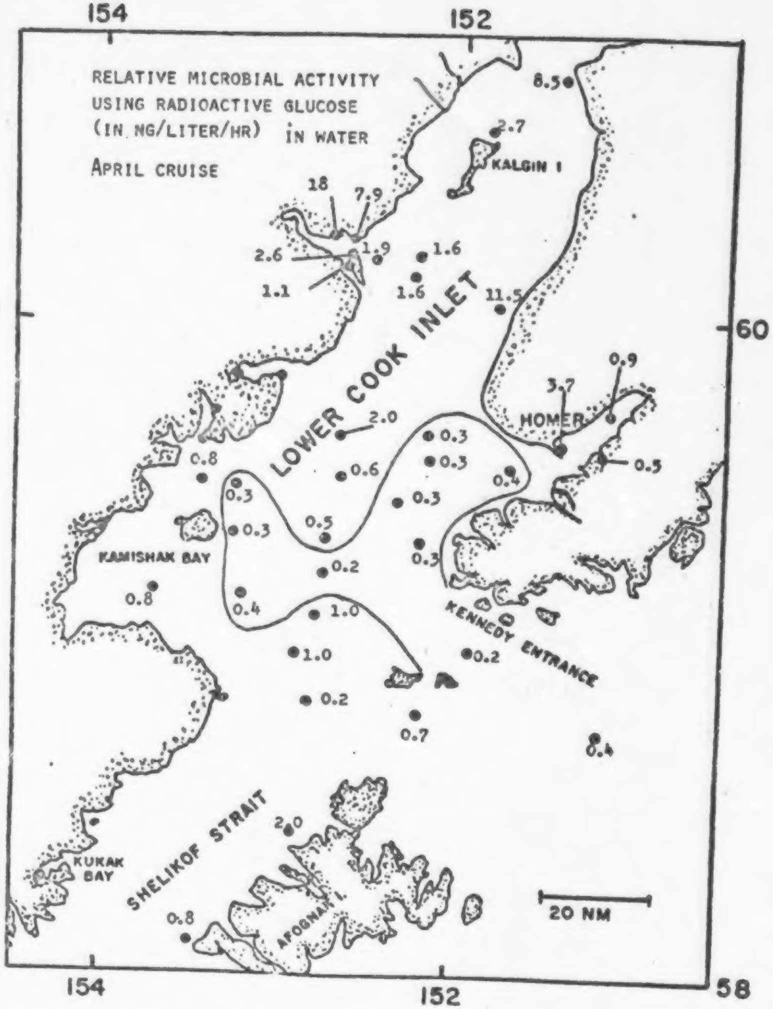


Figure 3. Relative microbial activity in water samples as measured using glucose during the April cruise. The units are reported as ng/liter/

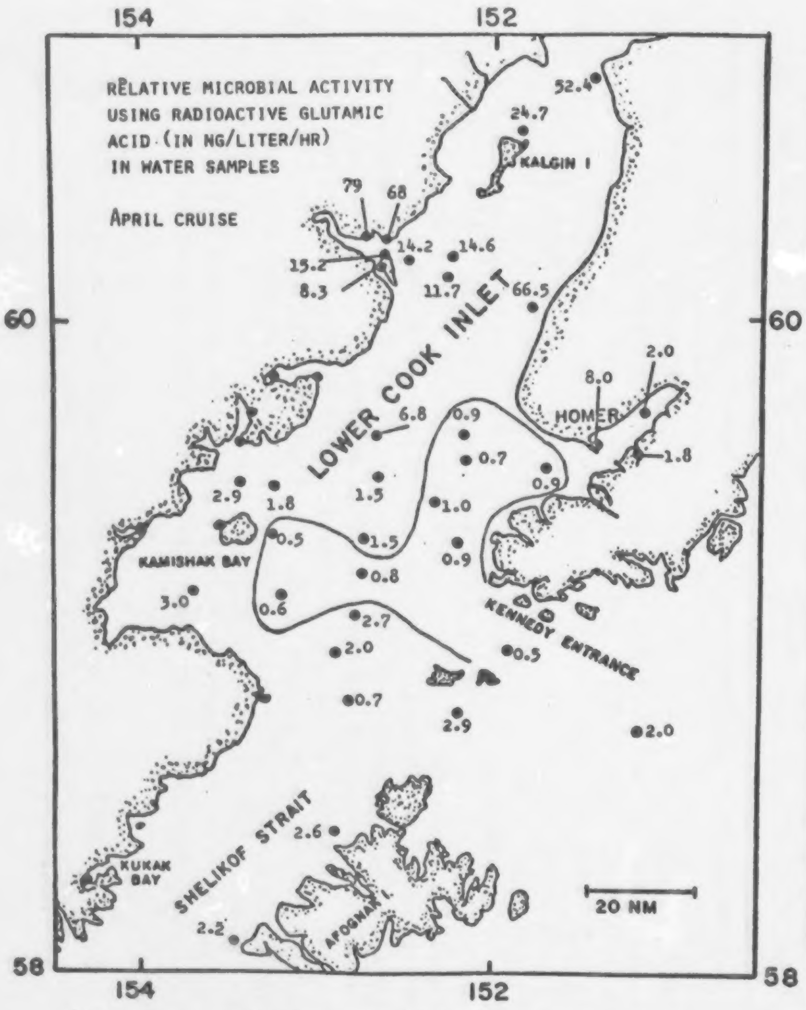


Figure 4. Relative microbial activity in water samples as measured using glutamic acid during the April cruise. The units are reported as ng/liter/h.

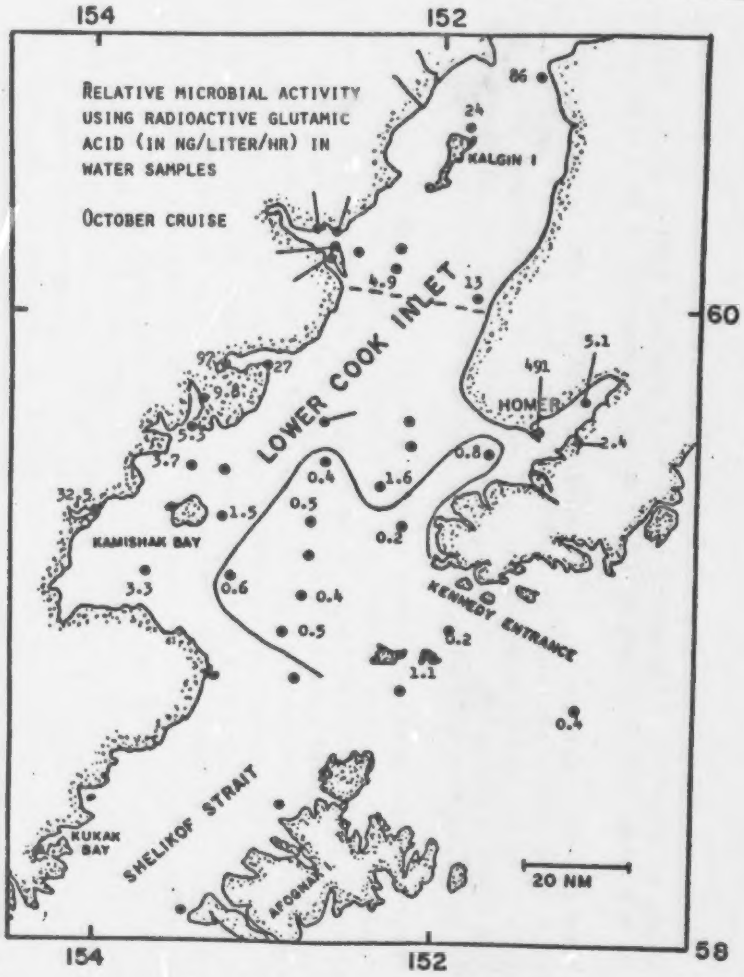


Figure 5. Relative microbial activity in water samples as measured using glutamic acid during the October cruise. The units are reported as ng/liter/h.

Cook Inlet is starting to produce an overall picture that will be valuable in managing the development of crude oil reserves in this region.

From the data presented, it is obvious that the forces acting in the Lower Cook Inlet can only be understood in terms of the broader factors acting on the entire region. If we had restricted our study to only the Lower Cook Inlet, we would not have been able to interpret our data in terms of the wider patterns which directly affect this area.

There are two distinct water masses present in the Cook Inlet: one to the north that is very turbid and of relatively low salinity and one to the south and southeast which is more typical of the open ocean water. We have found that both of these water masses have characteristic patterns of microbial activity and respiration. Glutamic acid uptake studies in surface water have shown that the relative microbial activity is very high and the respiration percentages are very low in the northern water mass. The reverse pattern is seen in the water mass to the south. Intermediate values were observed in regions where these two water masses meet in the area to the north and east of Augustine Island. This is the same region in which a gyre has been observed by other investigators. In general, the patterns of surface water microbial activity and respiration reflect the net surface circulation patterns reported by Miller and Allen (RU #436) (Environmental Assessment of the Alaskan Continental Shelf, Oct.-Dec., 1976 Principal Investigators' Reports, Vol. 3, page 782). Patterns of microbial activity appear to be related. The two water masses in question are clearly shown in Fig. 6 from surface salinity data. This same type of pattern has been reported by Kinney et al., 1969 (Prevention and Control of Oil Spills, API-EPA Proceedings, page 336). This is also the same type of pattern that one would expect from the current data presented by Miller and Allen (RU #436), (Oct.-Dec., 1976 OCSEAP Principal Investigators' Reports Vol. 3, page 782). Since these observations were taken at various times, it would appear that this is a relatively consistent phenomenon in this region. This same general pattern was observed in the relative levels of glutamic acid uptake in surface water microbial populations during both the fall and spring cruises (Fig. 4 and 5). If the surface current data observed by Miller and Allen were in effect during our observations, one would expect this water to have a net movement to the southwest, through Kamishak Bay to the west of Augustine Island and to the south and southwest through the Shelikof Strait. The relative microbial activity in the surface waters follows the same pattern with values becoming lower as the water moves south. During this time, it is presumed that the highly active suspended matter is settling out of the water column. Our studies of microbial activity in the sediments indicate that most of this material does not settle out in the sediments of Kamishak Bay but that it is deposited in the sediments of the Shelikof Strait.

When seasonal variation are compared in water samples taken from common offshore stations, there were very few differences seen in the resulting data. The average relative microbial uptake was somewhat higher in the spring when measured with glutamic acid but was about the same with glucose. These data suggest that there might be a slightly different type of organic nutrient available in the spring than in the fall.

There were also differences in the pattern of microbial activity in the interface between the two water masses. It is not known at this time if this is a seasonal phenomenon or if these differences reflect much shorter term fluctuations.

The average value for glutamic uptake was about the same during both seasons if the atypical values observed at Homer are omitted from the average. The uptake of glucose was again somewhat lower in the spring. This may reflect a decrease in the amount of carbohydrate available to the spring microbial populations. It is not known at this time what these differences mean in terms of the interactions between the microbial populations and crude oil.

Of perhaps equal interest were some of the differences seen at specific locations between the two seasons. The uptake of both glucose and glutamic acid was exceptionally high in the water samples taken in the Homer boat basin in the fall (five times greater than any other samples tested). During the spring sampling, the level of microbial activity was much lower for this station and the other stations sampled in Kachemak Bay. These differences probably reflect differences in the amount of organic material that was being released into the bay from Homer at these two times of year.

The heterotrophic potential method was employed to determine the microbial activity in the intertidal region. Table 1 illustrates that there is a considerably greater activity in the intertidal region compared to water samples taken 10 meter offshore. Again this may be the result of the availability of organic matter in the two different areas.

The respiration (mineralization) percentages for glutamic acid shows a similar pattern to the heterotrophic activity values (Fig. 7 and 8). The low values associated with the northern water mass are replaced with higher values in the waters to the southwest as the water moves through Kamishak Bay. The high values to the southeast reflect the influx of ocean water pushing into the inlet moving from the southeast to the northwest. Intermediate values were observed at stations #206, 225 and 236 in areas in which mixing between these two water masses probably takes place.

When one compares the patterns resulting from the glutamic uptake data and the respiration percentage data, there are some differences. Of these two sets of data, the respiration percentages more closely follow the surface salinity data than does the uptake data. The most

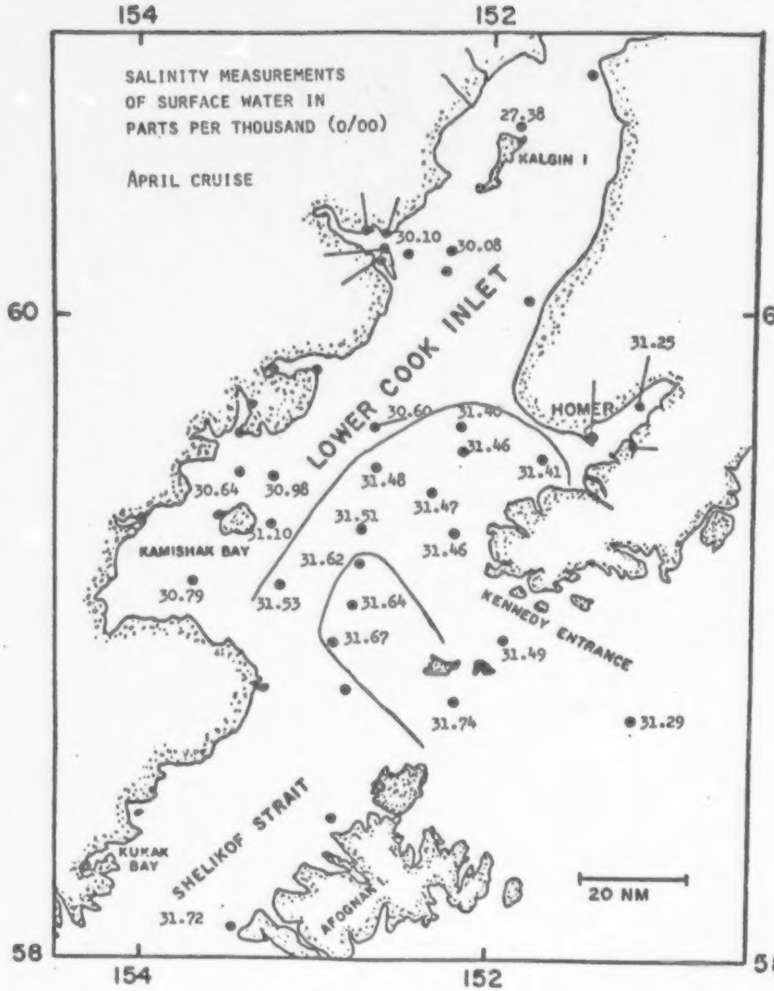


Figure 6. Salinity measurements made in surface water samples analyzed during the April cruise. The unit used is parts per

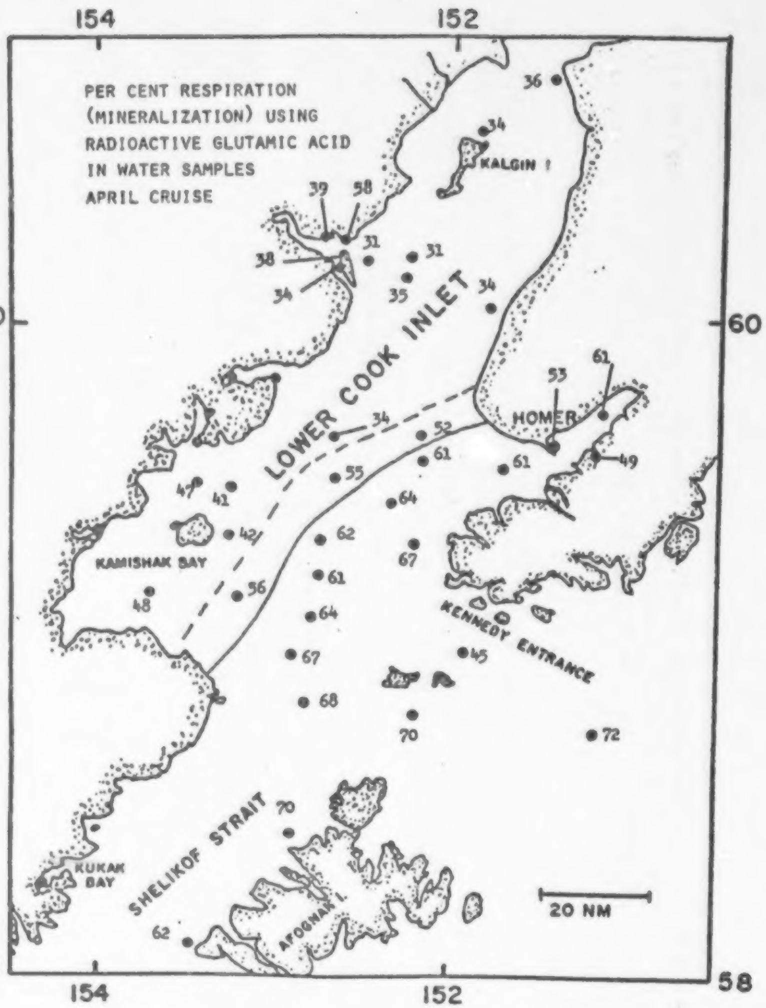


Figure 7. Respiration percentages observed in water samples as measured using glutam acid during the Apri

Table 1. A comparision of glutamic acid uptake in water samples taken in the surf zone and 10 meters offshore.

Glutamic acid uptake (ng/l/h)	
Intertidal	Offshore
14	4.9
26	5.8
39	21
8.3	0.9
256	236
51	50
3.5	2.1
7.3	0.4
3.3	2.6
2.4	1.1
40	18
6.7	3.7
1.0	0.5

probable explanation for this phenomenon is that the respiration percentages reflect the types of nutrients available to the microbial population. Under many conditions, the respiration percentages are inversely related to microbial activity (the correlation coefficient typically runs from about -0.4 to -0.5). This relatively low correlation reflects at least two possible situations which would cause an exception to this relationship. It is possible to have a population which has all of the nutrients it requires for growth but the nutrients are available at very low concentrations thus the microbial activity would be low and the percent respiration would also be low. The reverse situation can also occur, namely, there might be a deficiency in one or more growth factors in a water sample but in general, the levels of other available nutrients is high resulting in a high level of activity and high respiration percentage. It is also possible that the level of respiration observed in a given sample reflects the types of organisms present rather than their physiological state. The taxonomical studies being conducted by Dr. Atlas may help to clarify this point.

Even with these discrepancies in detail, both sets of data show that there is an area of mixing in the general region of the "null zone" which has been described by Miller and Allen.

The correlation that we found between suspended matter load and microbial activity suggests that the tidal currents may play a role in determining the level of microbial activity observed in a given location at a given time. After analyzing our data from the fall cruise, we concluded that the tidal currents were not a significant factor in affecting microbial activity. We now have data that suggests that the tidal state may indeed be a factor, particularly in relatively shallow regions where sediment can be introduced into the water column by mixing. Water samples taken at stations U and V in Tuxedni Bay illustrate this point. These stations were taken during a strong flood tide. Station U was taken near the mouth of the bay and V one mile further up the bay. The water flowing from station U to station V was flowing over a rather extensive mud flat area. The uptake observed at V was more than twice that observed at station U.

We found the relative microbial activity in the waters to the north was very high even though very little primary productivity is taking place in the area. This high activity was directly related to the suspended particulate load in these waters. As the tidal current velocities decrease to the south in the wider portions of the inlet, the surface water become less turbid and presumably, the particles are starting to settle out of the water column. We are curious as to whether this highly active suspended matter found in the northern water mass settles. An analysis of microbial activity in the Cook Inlet sediments indicate that little of this material settles out into the sediments of this area but rather is deposited in the sediments of the Shelikof Strait. The high microbial activity sediments of the Shelikof Strait undoubtedly is very important to the whole food chain in this region.

If the studies of crude oil--suspended matter interactions currently being conducted by Drs. Joel Cline and Richard Feely (PMEL) show that significant quantities of crude oil can become associated with these particulates, it is quite likely that crude oil would be introduced to these very active sediments of the Shelikof Strait.

It should be realized that the environment of the areas studied is cold. In actuality 90% of the marine environment is 5 C or colder. When we consider the Arctic environment, then we must taken temperature into consideration. Employing the heterotrophic potential method we have examined the effect of temperature on the activity of the in situ microbial population. We have observed that activity does occur at temperatures from which we take our samples but not as well as at elevated temperatures. Microbiologically we have two types of thermal groups present.

Morita (Bacteriol. Rev. 39:144, 1977) has redefined the term "psychrophile" (cold-loving bacteria) so that it includes organisms having an optimal temperature for growth at 15 C or lower, a maximal temperature of growth at 20 C or lower, and a minimum temperature for growth at 0 C or lower. Psychrotrophic bacteria are organisms capable of growth at 5 C or below (Eddy, J. Appl. Bacteriol. 23:189, 1960). Unlike some of the Antarctic water samples, the microbial populations in the Arctic do not have low maximum temperature for activity (see Gillespie, Morita, and Jones, J. Oceanogr. Soc. Japan, 32:74, 1976). This strongly suggests that we are dealing mainly with a psychrotrophic microbial population. Nevertheless in terms of activity of microbes in the water column, the percent respiration (mineralization) is one of the best microbial activity samples (measured by the heterotrophic potential method at -1.0 C) gave a value of 9.8×10^3 μg CO_2 produced/liter/nautical miles^3/day. If one extrapolates these values and takes into account the volume of the oceans, the figure would indeed be very large. An extrapolation of the amount of ammonia deaminated from glutamic acid was also made and the figures are 7.41×10^{-3} μg of ammonia released/liter/h which can be extrapolated to a figure of 1.9×10^9 g/nautical mile^3/day (Morita, Geesey and Goodrich, In Colwell and Morita, Effect of the ocean environment on microbial activity, Univ. Park Press, p. 386, 1974).

However, certain bacterial isolates from Arctic environments do have cardinal temperatures that fit the psychrophile definition. This includes some hydrocarbon degraders.

Because of the ability of microorganisms to function in the cycle of matter, Russell-Hunyer (Aquatic Productivity, McMillan Co., 1970) stated "if all the decomposing bacteria ceased their activities for only three months, then nearly all green plant production on this planet would cease almost immediately, and no higher animals (including man) could survive the temporary interruption of bacterial recycling."

At the time of this study, there were no data available on the in situ rates of nitrogen fixation in the Cook Inlet sediments. In fact, very little is known generally about this primarily bacterial function in marine sediments. It was our intention to evaluate the extent of nitrogen fixation in these sediments and to initiate experiments which would evaluate the effects of Cook Inlet crude oil on this process. The geographical variations in nitrogen fixation in the sediments collected during the spring cruise are given in Fig. 9. The highest rates were observed in Kachemak Bay with somewhat lower values observed at stations 395 and 388.

From the values observed for nitrogen fixation in Kachemak Bay, we calculated the annual rate of nitrogen fixation in the sediments of this bay. This calculation was made based on several assumptions which were: (1) that the average level of nitrogen fixation is 1 ng/g dry wt. sediment/hr, (2) that most of the nitrogen fixation is taking place in the top two cm of the sediment, and (3) the area of Kachemak Bay was 100 sq miles. Within the framework of these assumptions, the amount of nitrogen fixed annually in the sediments of Kachemak Bay would be $2x10^4$ kg. If this was also utilized in bacterial biosynthesis, this would result in annual bacterial biomass of about $4x10^5$ kg bacteria (roughly 400 tons). Presumably, this biomass would be available to higher trophic levels including species of commercial value. If this process was drastically altered by the presence of crude oil in the sediments, effects at all trophic levels might be seen.

Preliminary data has been obtained on the effect of crude oil, and energy source (sucrose) on the nitrogen fixation capacity of sediments obtained from the Lower Cook Inlet and the Beaufort Sea (Tables 2 and 3). In each case an added energy source increased the nitrogen fixing capacity of the microbes in the sediments. Generally the addition of crude oil to the system decreased the nitrogen fixing capacity of the indigenous microbial population in the sediments. However, much more experimental research must be done to complete the picture as to how crude oil affects the process.

The average value for bacterial concentrations in the sediments was $2.5x10^9$ bacteria per g dry weight sediment. This is lower than the average value of $4.9x10^9$ observed in the sediments during the October cruise. The average concentration of bacteria in the water samples analyzed was $3.0x10^6$ cells/ml. The average value for water samples taken from common stations during the October cruise was $1.1x10^6$ cells/ml. This is roughly twice the concentration observed in water samples taken from the same stations during the October cruise.

Dr. Atlas reported that both direct counts and viable plate counts indicate that the number of bacteria present in the water is lower in the winter than in the summer. This observation is supported by our measurements of relative microbial activity. He also reported that the plate counts indicated that the bacterial concentrations were higher in

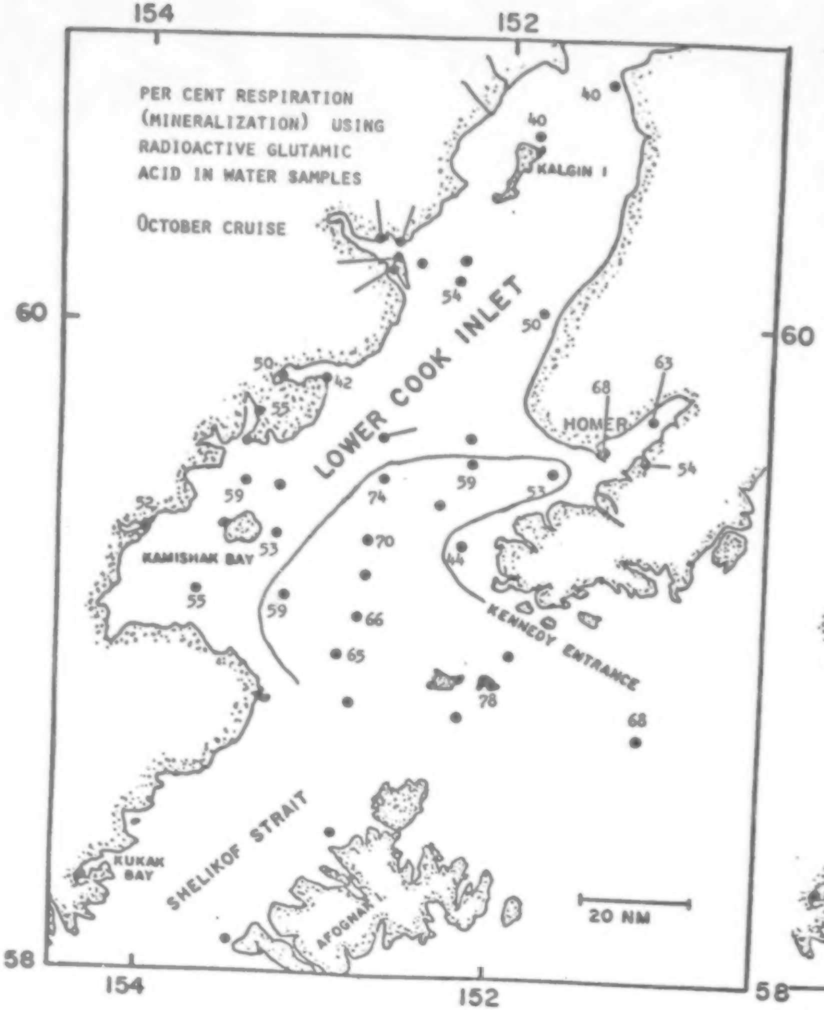


Figure 8. Respiration percentage observed in water samples as measured using glutamic acid during the October cruise.

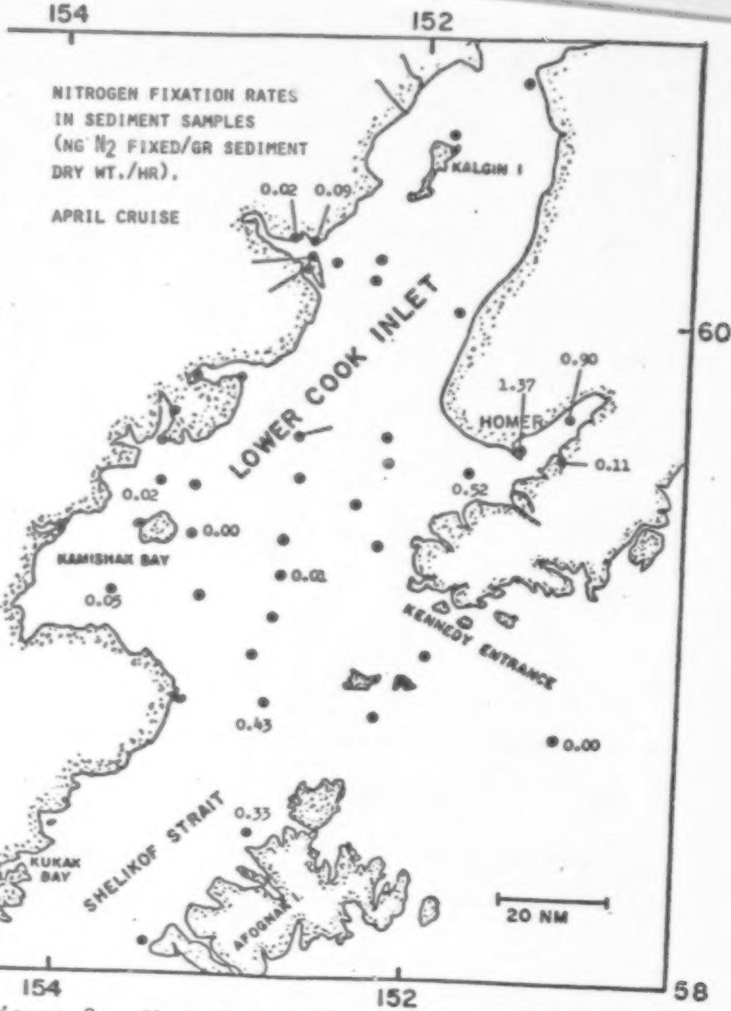


Figure 9. Observed rates of nitrogen fixation in sediment samples collected during the April cruise. The unit of measurement used was ng nitrogen fixed/gram dry weight of sediment/h.

Table 2. PRELIMINARY RAW DATA ON THE EFFECT OF CRUDE OIL ON NITROGEN FIXATION BY BACTERIA IN SEDIMENTS FROM THE BEAUFORT SEA. INCUBATION WAS FOR 7 DAYS AT 5 C.

SAMPLE NO.	WITHOUT SUCROSE		WITH SUCROSE	
	NO OIL	OIL	NO OIL	OIL
301	60	63	91	65
302	95	95	146	146
303	47	40	62	53
306	12	15	362	143
308	48	45	57	63
314	6	6	202	245
315	10	12	42	26
317	3	3	179	79
319	29	30	480	381

ALL VALUES IN PICO MOLES

Table 3. PRELIMINARY RAW DATA ON THE EFFECT OF CRUDE OIL ON NITROGEN FIXATION BY BACTERIA IN SEDIMENTS FROM THE LOWER COOK INLET. INCUBATION WAS FOR 10 DAYS AT 5 C.

SAMPLE NO.	WITHOUT SUCROSE		WITH SUCROSE	
	NO OIL	OIL	NO OIL	OIL
411	66	86	5726	2194
425	203	242	4233	7343
440	163	126	1095	1494
444	149	133	250	199

ALL VALUES IN PICO MOLES

the sediments studied in the summer of 1975 than in the average winter of 1976. Our direct count data reflects this same trend but the average relative level of microbial activity was one order of magnitude lower in the winter sediment samples. It is quite likely that bacteria were selectively removed from the water column during the freezing process and were still viable but also relatively inactive.

In addition, he reported that the average concentration of bacteria in the sediments was much higher in the samples analyzed in the summer of 1976 than those analyzed in the summer of 1975. Our relative microbial activity data reflects the same trend.

A high rate of activity (as indicated by a high V_{max}) is indicative of a high rate of bacterial growth (increase in number of cells). Although the direct counts do not reflect a high number of bacterial cells in the winter, it should be recognized that the bacterial cells are being eaten by the meiofauna very rapidly. There are many filter feeders in the marine environment as well as certain meiofauna that eat bacteria directly. Decaying phytoplankton as well as other detrital particles are extensively colonized by bacteria, which in turn act as a high-nutrient food source for a wide variety of marine animals (Morita, In Anderson and Zahurance (eds.), Oceanic Sound Scattering Prediction, Plenum Press, p. 445, 1977). It should also be recognized that bacteria are much more nutritive than phytoplankton (see Objectives and Rationale). This area of microbiology must still undergo examination (see Future Research Problems).

Many of the sediments that were collected were analyzed for the presence of presumptive Desulfovibrio sp. Only one sediment (Station #214) showed no evidence of these organisms at the lowest dilution used (10-3). The other sediments all showed growth of these organisms at this dilution and/or at higher dilutions. The estimated concentrations of these organisms are shown in Figure 10. The highest concentrations observed were seen in Tuxedni Bay beach sediments, station K at Homer, station 227 in the Cook Inlet, and station 388 in the Shelikof Strait.

Of potential interest to those planning to construct metal structures that come into contact with sediments containing a group of anaerobic bacteria known as Desulfovibrio is the fact that the growth of this organism may produce considerable corrosion of most metals. Desulfovibrio has isolated from Beaufort Sea sediments also. These organisms are also known to grow in drilling muds with the resulting release of H_2S. Except for the sediment analyzed from station 214, all samples tested showed significant concentrations of this organism in Cook Inlet sediments (10^3 to 10^6 bacteria per ml sediment). In most dilution tubes, growth was visible within four weeks incubation at 8 C. Desulfovibrio is also known to cause clogging of oil bearing strata when water injection is used in secondary recovery. Both corrosion and plugging oil strata by Desulfovibrio is being experienced in the North Sea Drilling platform operations.

A study was designed to see if there were any correlations between crude oil degradation potentials in sediments (Fig. 11) and the uptake of glutamic acid, glucose, or acetate in the same sediments. This study was initiated because we did find a correlation between crude oil degradation potentials in surface waters with glucose uptake rates during the October 1976 Cook Inlet cruise. We did not find any significant correlation between any of these parameters. A correlation coefficient of 0.57 was found to exist between crude oil degradation and the ratio of glutamic acid uptake to glucose uptake in the same samples.

PROBLEMS ENCOUNTERED

Thanks to the excellent logistical supported by the Juneau regional office, no serious logistical problems were encountered. Our main problem again centers around the lack of sufficient funds to provide the manpower required to continue our work on hydrocarbon utilization by bacterial isolates taken from the Cook Inlet. The personnel currently available under the present funding schedule are occupied primarily with field study obligations. Because of the long term nature of the work that must be done, enough manpower must be available to insure that these studies are continued at Oregon State during the field operations. Funds have just been received (6/13/77) to remedy this situation.

RECOMMENDATIONS FOR FUTURE STUDIES

We recommend a continuation of our current studies into other lease areas as well as to complete our studies in the Beaufort Sea as well as the Lower Cook Inlet so that a seasonal picture of the microbial involvement in these waters can be obtained. Further studies on crude oil--microbial relationship should be carried out--especially employing crude oil from the various lease areas.

One of the missing areas of study in the entire biology of the Arctic is the detritus--microbial relationship in the food chain. In this study we must recognize that the bacteria replaced the phytoplankton in the first trophic level, especially during the winter months when the amount of sunlight is diminished. It has been shown that in estuarine environments that the bacteria are the main source of food for the meiofauna. Now is the time to develop laboratory techniques for field studies dealing with this problem.

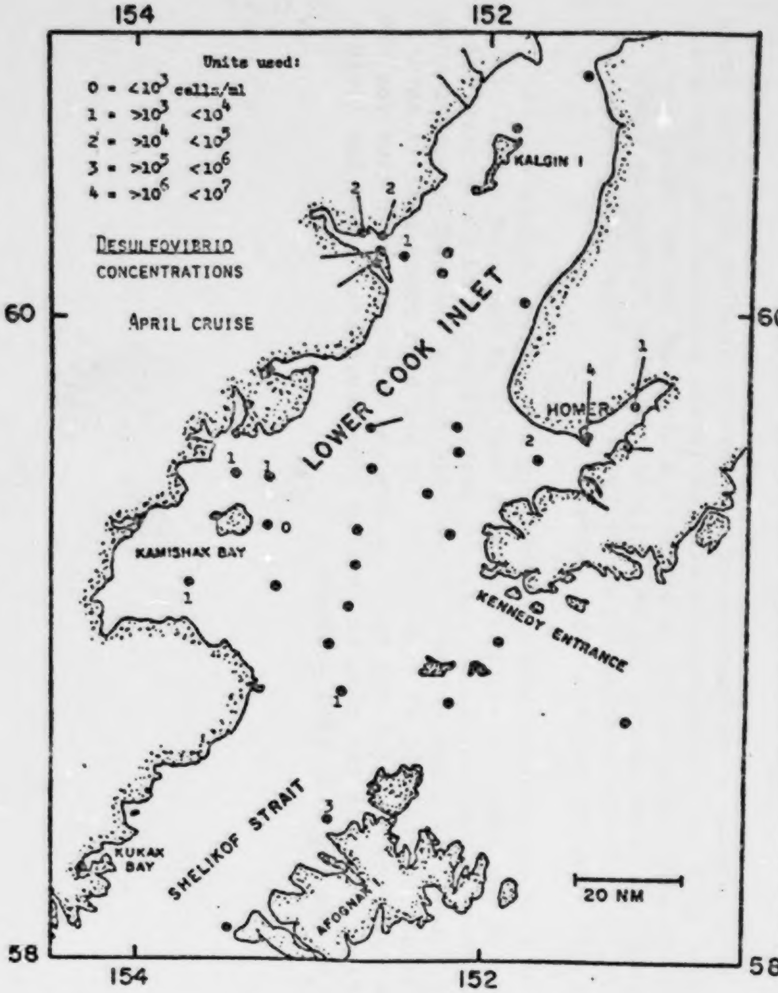


Figure 10. Relative concentrations of Desulfo-vibrio sp. present in sediments analyzed during the April cruise.

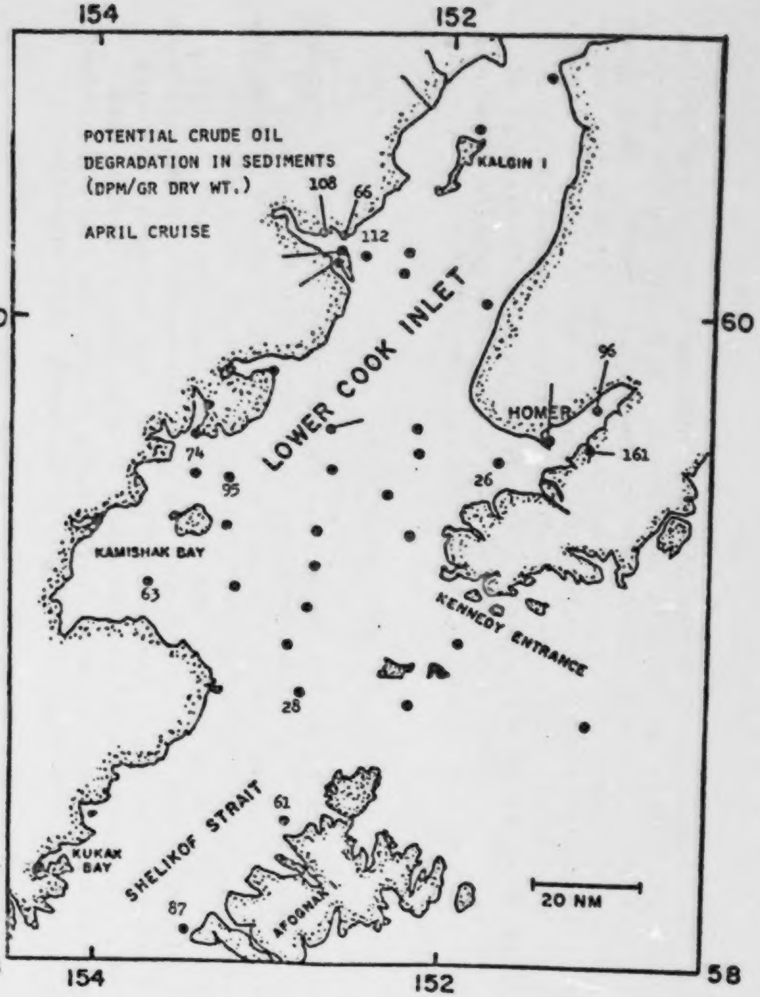


Figure 11. Crude oil degradation potential measurements in sediments collected during the April cruise. Units reported as DPM/ dry weight.

PUBLICATIONS

Morita, R. Y., R. P. Griffiths, and S. S. Hayasaka. 1976. Base-line study of microbial activity in the Beaufort Sea. Abstr. Ann. Mtg. American Society for Microbiology, p. 117.

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9. INFLUENCE OF PETROLEUM ON EGG FORMATION AND EMBRYONIC DEVELOPMENT IN SEABIRDS

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OBJECTIVES

This study is an attempt to appraise the effects that a brief exposure to an oil spill would have on the reproduction of Cassin's Auklets (Ptychoramphus aleutica) and on Western Gulls (Larus occidentalis) nesting on the Farallon Islands of California. These species are representative of many birds breeding along the Pacific Coast that are at risk from oil pollution during the reproductive period.

Females of breeding pairs of auklets dosed with bunker C oil or empty capsules as controls, and egg production, hatching success, and fledging success were determined. Samples of eggs were compared in regard to yolk structure, presence of oil components, and embryonic development, and were related to observations made previously at UCD with Japanese quail.

Attempts to dose gulls were unsuccessful.

Methods of detecting oil components in egg yolk have been developed in preparation for further field studies of bunker C and Prudhoe Bay crude oil effects on auklets.

Investigators in the field work on the Farallon Islands were Steve Morrell (PRBO), Tom Roudybush (UCD), Ron LeValley (PRBO) and Joan Dobbs (UCD); in the laboratory work, Tom Roudybush and Tom Wootton (UCD).

CASSIN'S AUKLET STUDY

During the 1977 breeding season on Southeast Farallon Island, 251 female Cassin's Auklets (Ptychoramphus aleutica) were force-fed gelatin capsules that were empty or contained 300 mg or 600 mg bunker C oil. These birds and their eggs and chicks were observed throughout the breeding season, as were birds in a control group. No significant difference in laying, hatching or fledging success were observed among the four groups.

METHODS

In January and early February 1977, 300 Cassin's Auklet burrows were selected and altered to allow human access. An additional 80 control burrows, studied since 1972 and in which birds were not given oil or capsules, were used to compare the effects of force feeding. Beginning in mid-march, all burrows were checked every other day until the first egg was found. On the night of 31 March we began our program of dosing at least 30 female auklets per night.

Each study burrow was checked until a pair of birds was found. We sexed the pair by measuring the bills with calipers. This method was proved reliable by analysis of specimens in museums. If the bill lengths were not distinctly different (an uncommon event), the bill depth was also measured. The bird with the smaller bill of each pair was assumed to be the female. She was force fed one of three possible experimental doses -- an empty capsule or a capsule containing 300 mg or 600 mg bunker C. Both members of the pair were then returned to their burrow. The type of dose was alternated in sequence (600 mg, 300 mg, empty capsule) so that equal numbers of each treatment were administered each night. Dosing was completed on 7 April after eight successive nights of work.

All experimental burrows were checked daily for eggs through 14 April, after which they were checked every four days. The control burrows were checked every other day throughout the study. When an egg was found, the burrow was not checked again for 38 days (to determine incubation period and/or hatching weight) or 42 days (to determine hatching success). Hatching weight was measured to the nearest 0.5 gram with a Pesola spring scale which was checked daily for accuracy with an electronic balance. After hatching, at least 10 chicks from each group were weighed daily (when alone) until fledging, others were weighed beginning shortly before fledging and others were checked only to determine fledging success. In all comparisons of times, weights and success, only the first egg laid is considered and only if it was laid on at least the second day after a selected experimental dose was administered.

The following definitions apply to this study:

Incubation period is the first day the egg is found until the day before the chick is found free of its egg shell;

Nestling period is the first day the chick is found until the chick is fledged;

Hatching weight is the weight of a chick on the first day it is found (within 24 hours of hatching);

Fledging weight is the weight of a chick on the last day it is in its burrow before fledging;

Maximum number of eggs possible equals the number of females dosed minus the number of burrows that collapsed before an egg was laid;

Maximum number of eggs possible to hatch equals the number of eggs laid minus the number of eggs collected and the number of burrows collapsed, filled in, or where checking was discontinued.

RESULTS

The data for breeding success in each of the four groups are summarized in Table 1. Chi square tests of the four groups in all possible combinations of two showed no significant differences between any two groups for either hatching or fledging success.

No differences were apparent in timing of egg laying following treatment for any of the three treatment groups (Table 2). However, when date of egg laying is considered (Table 3), there appeared to be a slight delay in onset of laying as the oil dosage was increased. This cannot be considered significant. Egg laying in the group given empty capsules and in control group were similar. The first egg for the control group was laid on 3 April, while the first eggs (after treatment) for the three groups dosed were -- empty capsule, 3 April; 300 mg, 5 April; 600 mg, 2 April (second egg in this group laid 7 April). The discrepancy in onset of egg laying disappeared within the first two weeks.

DISCUSSION

An examination of Table 2 shows that most of our attention to hatching and fledging success, period and weight was directed to the group of eggs laid 2-7 days after dosing. Since yolk formation in the Cassin's Auklet eggs take eight days (Roudybush and Grau, 1977), we felt that the effects of various treatments would be most apparent in this group. However, no major differences were found. The incubation periods, fledging time and fledging weights are comparable in all groups and are also comparable with the findings of Manuwal (1972) from his studies on S.E. Farallon in 1969-71: Mean incubation period--37.8 days (range 37-42; n=86); fledging time--41 days; fledging weight--158 grams. The hatching weight data for the three treatment groups are also comparable with each other and with Manuwal's figure of 21 grams.

Although there appeared to be a slight delay in the onset of egg laying with increased oil dose (Table 3), the effect cannot be considered significant. Delayed egg laying can be important to breeding success, however, since late breeders always do very poorly (PRBO unpublished data).

Table 1. Breeding success of treated and control Cassin's Auklets - 1977

	Empty gelatin capsule	300 mg Bunker C	600 mg Bunker C	control
# of females dosed before first egg laid	86	84	81	-
maximum # of eggs possible to be laid	82	75	78	79
Total # of eggs laid	80	72	77	75
% eggs laid of possible maximum	98	96	99	95
# eggs laid 2-7 days after dosing	21	27	25	-
% eggs laid 2-7 days after dosing	26	38	32	-
maximum # of eggs possible to hatch	74	68	66	74
total # of eggs hatch	51	57	48	62
% eggs hatch	69	84	73	84
maximum # of 2-7 day eggs possible to hatch	19	26	23	-
# of 2-7 day eggs hatch	13	22	17	-
incubation period (days)	*38.9±1.6 N=10	*38.5±0.8 N=16	*38.9±1.2 N=12	38.4±0.9 N=55
hatching weight (grams)	*18.5±1.4 N=9	*20.0±1.9 N=14	*18.5±2.4 N=10	-
fledging time (days)	*43.0±3.8 N=9	*42.0±2.3 N=11	*44.0±1.9 N=11	41.0±3.1 N=48
fledging weight (grams)	*153.0±10 N=9	*149.0±12 N=12	*150.0±13 N=11	152.0±14 N=48
% fledging success	*90.0 N=10	*78.0 N=18	*85.0 N=13	87.0 N=55

* Data are for eggs laid 2-7 days after dosing only.

Table 2. Timing of egg laying by Cassin's Auklets following treatment

Dose	Total # eggs laid		Number of days between dosing and egg laying 2-7	8-13	14-19	20-25	26-31	32-37	38+	Un kn
Empty capsule	80	# of eggs	21	22	19	4	7	1	4	2
		% of total	26	28	24	5	9	1	5	3
		cumulative %	26	54	78	83	92	93	98	
300 mg Bunker C	72	# of eggs	27	18	12	8	1	3	0	3
		% of total	38	25	17	11	1	4	–	4
		cumulative %	38	63	80	91	92	96	96	
600 mg Bunker C	77	# of eggs	25	17	15	8	4	2	2	4
		% of total	32	22	20	11	5	3	3	5
		cumulative %	32	54	74	85	90	93	96	

Table 3. Cumulative percent of total number of first eggs laid by auklets

	Empty capsule (N=80)	300 mg Bunker C (N=72)	600 mg Bunker C (N=77)	Control (N=75)
April 1-2			1	
3-4	3			1
5-6	4	6		8
7-8	8	17	7	11
9-10	14	32	25	18
11-12	27	42	39	34
13-14	38	52	44	45

The procedures of bill measurement, dosing and checking for egg laying apparently did not cause birds to abandon their burrows, nor to affect experimental birds in other ways, when done in the manner used by us. All sixty birds (30 pairs) handled the night of 31 March were banded with numbered aluminum leg bands. Seventeen of those 30 burrows were checked on the night of 16 June. Of the 34 adults that could have been present in the 17 burrows, 29 adults were found in 16 burrows; only one burrow was vacant. Of the 29 adults, 28 were birds originally treated and banded. The one unbanded bird was with a banded female that had produced an egg that did not hatch. These data indicate that the auklets can tolerate well the level of disturbance to which we exposed them.

Some burrows in loose soil were destroyed by our activity. We also suspect that we caused some abandonment of eggs when we made repeated checks to determine exact date of hatching and hatching weight.

The incubation periods and hatching weights did not differ significantly among the groups. Samples of eggs that failed to hatch were taken to Davis for examination of egg contents. Most of the embryos were macerated or decomposed. No pattern of causes of death was found.

Definitive chemical analyses of auklet eggs for presence of oil components have been delayed until techniques are developed using quail and other laboratory birds, as detailed later in this report. Preliminary data indicate, however, that no sigificant amounts were incorporated into auklet eggs.

WESTERN GULL STUDY

The field observation on the effects of the oil on gulls confirmed the 1976 results in which dosing became the principal problem. In the present year, capsules containing oil were coated with hydrogenated vegetable oil to lengthen time of capsule solution, and the capsules were sewn inside small squid. The gulls reaily took the squid. As soon as the capsule dissolved and the oil was released, the squid, now covered with oil, was regurgitated, and was eaten by the mate or another bird. Within a few seconds it was regurgitated again. Sometimes this was repeated several times. This study convinced us that dosing gulls by capsules is fruitless. Other small trials with gulls included snaring and smearing with oil on the head and neck and injecting the oil intraperitoneally into two birds. All these gulls abandoned their nests. Another potentially more useful technique that is still being evaluated was to coat the first egg laid with oil, and after the female incubated it overnight thus coating the brood patch with oil, the egg was removed. Eggs laid subsequently were collected for chemical analysis and observation of yolk structure. Results are not yet complete, but they do not appear to be promising.

FATE OF INGESTED OIL

Following the observations of gull behavior in regurgitating oil, auklets and other birds were also studied. To determine the time which is required for elimination of oil from the guts of auklets, birds were captured at random at night and place in cardboard boxes covered with netting. The auklets were fed 600 mg of bunker C oil and observed until the oil passed in their feces. The auklets began eliminating the oil at a mean time of 36 minutes after being fed (Table 4).

Adult Japanese quail reared in cages and adult pen-reared mallards deprived of food for 16 hours (overnight) were compared in their responses to oil with fed birds, with the results shown in Table 4. It will be noted that fed quail took a mean of 177 minutes before eliminating oil while unfed quail took only 29 minutes. Mallards responded differently: feeding did not affect passage time.

Black-footed Albatrosses and Common Murres that are being cared for in preparation for release were also dosed. The numbers are small, and two of the albatrosses were found later to be infected by bacteria, but their responses appeared to be different from the other birds studied. The murres reacted violently to the oil dose.

If the same relationship between oil retention times in fed and unfed quail holds for fed and unfed auklets, fed auklets would be expected to retain oil for three hours before beginning to eliminate it. This would greatly extend the time of the auklet's exposure to oil and would expose the auklet at a time when it is actively absorbing nutrients. This may increase the toxicity of oil to both the auklet and any egg which is forming at the time of exposure.

STRUCTURAL AND CHEMICAL EXAMINATION OF YOLKS

Auklet eggs collected during the critical period of 2-7 days after dosing with bunker oil were frozen, fixed in formalin, and examined for structural changes such as those observed in eggs laid by quail after being dosed with bunker C (Grau et al., 1977). No characteristic features could be detected. At that time, before hatching data were obtained, we had the first indication that auklet responses were different from those of quail given comparable doses of oil. This lack of effect spurred our interest in developing chemical methods that would detect hydrocarbons or metabolites of petroleum compounds in eggs if they are present, and quantifying the amounts present.

Eggs of quail and chickens that had been given single doses of bunker C oil were treated and extracted, and various techniques were used to detect compounds that might be unique to petroleum-fed birds. The richness of egg yolk in triglycerides, phospholipids, sterols, and pigments complicates the extraction and treatment processes, and makes

Table 4. Responses of several bird species to dosing with capsules containing bunker C oil

Species	Number of Birds	Time between dosing and			
		Regurgitation		Fecal Elimination	
		Mean	Range	Mean	Range
Cassin's Auklet[1] (*Ptychoramphus aleutica*)	10	-	-	36 min	12-82 min
Western Gull[1] (*Larus occidentalis*)	15	*	0-35 min	-	-
Common Murre[2] (*Uria aalge*)	3	10 min	7-13 "	18 min	7-33 min
Black-footed Albatross[2] (*Diomedea nigripes*)	3	-		10 hrs	6-20 hrs
Japanese Quail[3]	6 fed	-		177 min	143-226 mi
(*Coturnix coturnix japonica*)	5 unfed	-		29 "	11-67 "
Mallard[3]	6 fed	-		52 "	20-145 "
(*Anas platyrhynchos*)	6 unfed	-		46 "	31-79 "

*Upon contact with oil. Some oil was hidden in bait.

1) Dosed on S.E. Farallon Island.

2) Dosed at International Bird Rescue Research Center, Berkeley, CA.

3) Dosed at University of California, Davis, CA.

We observed by aspiration that no food was present in auklet stomachs at night. This information, together with data on passage time of oil in fed and unfed quail and mallards indicates that if auklets are similar to quail, then oil may have passed through the auklets too quickly to affect their eggs.

The cooperation of Alice Berkner of the International Bird Rescue Research Center is gratefully acknowledged.

difficult detection of hydrocarbons and their metabolites in extracts prepared from yolks.

Much of our laboratory work and all of our field work to date has utilized bunker C oil as the potential spill component. This material is recognized as being potentially harmful to birds because of its toxicity, viscosity, adhesiveness, and its ubiquitousness as a fuel oil for ships. It constitutes a signficant component of the petroleum in commerce.

From experiences in other laboratory (Ehrhardt, 1972; Neff and Anderson, 1975; Warner, 1976), we presumed that ultraviolet absorption and gas chromatographic analyses of saponified, Florisil-treated extracts would be the most likely methods of differentiating between eggs of control and oil-fed birds, but early attempts were not successful. The high lipid content of egg, and the presences of interfering substances thwarted these efforts to find oil components.

Yolks from oil-treated quail and chickens were found to differ from normal yolk in appearance and response to solvents. "Oil" yolks were lighter in color than normal. Denaturation and initial extraction of the thawed yolk with acetone, as part of phospholipid studies resulted in a stringy texture as compared with the normal crumbly appearance. No major differences were found in phospholipid distribution (by thin-layer chromatography) or by ultraviolet absorption of extracts containing primarily triglycerides or phospholipids.

Our most recent attempts to detect oil components in yok have been more encouraging. We are now able to detect differences in fluorescence of extracts prepared from "oil" and normal eggs after thin-layer chromatography of yolk extracts that have been cleaned with alumina and silica gel. The present method has not been tested extensively, and will no doubt be modified later. Differences have also been found in gas chromatographic analysis of these extracts, indicating that many petroleum hydrocarbons are incorporated into eggs.

METHODS

A 500 mg sample of frozen yolk was dried in a vacuum oven at 60° for 8 hours, or alternatively, a sample of yolk that has been fixed in 4% formalin after freezing (Grau, 1976) was used without drying. The sample was extracted by agitation in a beaker with 15 ml of petroleum ether, then with 15, 10, and 10 ml portions, and the extract was filtered into a flask. A mixture of 750 mg aluminum oxide G (E. Merck) and 750 mg silica gel (Absorbosil-3) both washed twice with petroleum ether, were added to the extract, shaken several times, filtered into a beaker, the solvent was evaporated, and transfer with isopentane to one spot on an activated silica gel plate scored with vertical grooves to prevent cross contamination. The plate was developed with isopentane: isopropanol: chloroform (100:1:0.2) and observed in ultraviolet light (253 nm).

RESULTS

Extracts from normal eggs showed some dark orange fluorescence at the front; those from quail or chickens fed bunker C showed a bright, light blue fluorescence. Spectrophotofluorometry of eluates of these spots is now being developed. Gas chromatographic analyses of extracts show differences between normal and "oil" yolks, but only preliminary results are presently available (GC work in collaboration with W. G. Jennings, Dept. Food Science and Technology, UCD).

In addition to the eggs from quail or chickens fed bunker C, fluorescence differences are apparent from Prudhoe Bay crude oil fed to quail, and Kuwait crude oil and South Louisiana crude oil fed to ducks (Duck eggs kindly submitted by W. N. Holmes, University of California, Santa Barbara, from mallards chronically fed 1% oil).

Several auklet eggs from dosed and control birds have been extracted and examined for fluorescence, but no differences have been found. Because the number of eggs laid during the 2-7 day post-dose period was low, the results cannot be considered as conclusive.

PROBLEMS ENCOUNTERED AND PROPOSED SOLUTIONS

The major problem in the auklet study was related to elimination of oil by the birds before effects could have been caused. Preliminary indications are that Cassin's Auklet do not retain petroleum in their bodies, and so apparently suffer no detrimental effects in terms of breeding success. However seabirds are far more likely to be exposed to petroleum while feeding at sea than while at their terrestrial breeding sites. In 1978, we therefore plan to feed some auklets small amounts of euphausiids (their normal diet) at the same time they are fed petroleum. The petroleum may be retained longer if food is also present in the stomach, as would be the case of birds exposed to oil while feeding at sea. We suspect that Cassin's Auklets coming ashore at night at the start of the breeding season may do so with empty or nearly empty stomachs.

The regurgitation responses of Western Gulls present such major problems that this part of the original plan will be abandoned for the present.

Although it is now possible to detect oil components in eggs, it is not possible to ascribe the various effects on reproduction to particular components of refinery products or of crude oils. Bunker C adversely affects egg production, shell quality, and embryo survival of quail (Grau *et al*., 1977) but Prudhoe Bay crude oil affects only aspects of reproduction other than embryo survival (Engle *et al*., 1977). Further studies of the effects of various fractions of bunker C embryos will be pursued, in order to provide baseline information that can be applied to wild birds at risk from potential oil spills.

The encouraging recent results with photofluorimetric and gas chromatographic analyses are expected to be improved by high pressure liquid chromatographic analyses, which will soon be applied to egg extracts with the collaboration of W. G. Jennings.

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10. EFFECTS OF PETROLEUM EXPOSURE ON THE BREEDING ECOLOGY OF THE GULF OF ALASKA HERRING GULL GROUP (*Larus argentatus* x *Larus glaucescens*)

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OBJECTIVES AND RATIONALE

This research is addressed to the following task: an analysis of the effects of petroleum exposure on the breeding ecology, including incubation behavior and hatching success, of the Gulf of Alaska Herring Gull group (*Larus argentatus* x *Larus glaucescens*).

The devastating effects of massive oil spills on seabird survival are widely reported, but little is known of the effects of chronic low-level oil pollution on avian reproduction (Grau et al., 1977). Previous studies of petroleum effects on seabird eggs suggest hatchability is markedly reduced (Gross; Birdhead et al., 1973; Patten and Patten, 1977). Rittinghaus (1956) and Hartung (1963, 1964, 1965) reported that marine birds contaminate eggs with oil from the environment and hatching success of eggs thus exposed to petroleum was markedly reduced even after extended periods of incubation. Abbott, Craig and Keith (1964) suggested that oil interfered with normal respiratory exchange through the eggshell, while Szaro and Albers (1976) found hatching success of eider (*Somateria mollissima*) eggs was significantly reduced by microliters of petroleum exposure, that is, very small quantities. Patten and Patten (1977) have recently found unweathered North Slope Crude Oil 22 times more toxic than equivalent amounts of mineral oil under field conditions. Grau et al. (1977) have confirmed in laboratory experiments that small amounts of bunker C oil significantly depress bird reproduction. Indeed, oil exposure has been used in the past to control gull populations along the Eastern Seaboard of the United States and in several western U.S. wildlife refuges (Gross, 1950; R. King, USF&WS, personal communication).

In summary, literature on the effects of oil exposure on the reproduction of marine birds is limited. Studies that do exist suggest high toxicity of petroleum to eggs, and marked effects upon the reproductive productivity of females. Complete knowledge of the effects of petroleum exposure in various forms is needed to evaluate and predict the full impact of oil pollution on the annual productivity of marine bird populations.

This current research is to provide information on the effects of North Slope Crude Oil on the hatching success and incubation behavior of key seabird species nesting on Alaskan barrier islands in proximity to Valdez tanker lanes and offshore oil lease areas.

Species examined are Herring Gulls (Larus argentatus) and Glaucous-winged Gulls (Larus glaucescens), which are common inshore and marine scavengers nesting in colonies. The study sites are the largest gull colony in the northeast Gulf of Alaska, Egg Island, located 10 km SE of Point Whitshed and 20 km south of Cordova (60°23'N, 145°46'W), and Dry Bay, 75 km SE of Yakutat (59°10'N, 138°35'W).

Our research objectives are thus defined as:

1) to determine threshold levels of petroleum effects to gull reproduction under field conditions, and

2) to test alteration of incubation behavior and ability to produce second (replacement) clutches following experimental mortality due to petroleum exposure.

MATERIALS AND METHODS

Our methods include analysis of reproductive productivity at a series of nest sites marked with survey flags. Petroleum exposure to eggs is by drops from microliter syringes with repeating dispensers, with equivalent amounts of non-toxic mineral oil applied to a control sample. Reproductive success and mortality are compared to further controls of 'normal' (unexposed) colonies adjacent to the experimental areas. The use of microliter syringes allows for precise manipulation of tiny amounts of petroleum exposure, which is in 20, 50, and 100 microliter doses at three (3) stages of incubation. Petroleum used in North Slope Crude Oil provided by NMFS Auke Bay Laboratory, with commercially available mineral oil as the control.

RESULTS

1) To determine threshold levels of petroleum exposure to gull reproduction, we conducted an oiling experiment in a colony at Dry Bay, mouth of the Alsek River, 75 km SE of Yakutat, Alaska. The oil experiment was carried out in an experimental area adjacent to controls known as 'A' and 'B' colonies. Receptors were a mixed group of Glaucous-winged x Herring Gulls (Table 1).

Table 1
Petroleum Exposure Parameters

Sample size	Dose	Date	Approx. Day of Incubation
10 nests 30 eggs	20µl	24 May	1
20 nests 60 eggs	50µl	1 June	8
20 nests 60 eggs	100µl	4 June	11

Our conclusions from the above experiments are: very small amounts of North Slope Crude Oil exposure to gull eggs in the field, at early stages of incubation, lead to high embryonic mortality (Tables 2, 3). Embryonic resistance to petroleum exposure increases with the duration of incubation (Table 4). Mineral oil in equivalent microliter doses causes no significant mortality (Table 5).

2) To test alteration of incubation behavior and ability to produce second (replacement) clutches following experimental egg mortality due to petroleum exposure, we conducted the following experiment: at Egg Island, the largest gull colony in the northeast Gulf of Alaska, located 20 km south of Cordova, we chose our experimental and control areas to coincide with our established study site (RU #96-76). There were 75 nests in the experimental area, compared to 186 in the adjacent 'normal' control colony. The experimental and control areas are located on the ocean slope of the stabilized meadow-covered dunes at the east end of Egg Island in proximity to the U.S. Coast Guard Light Tower (Figures 1, 2, 3).

Oil was delivered to completed clutches of three eggs at the tenth day of incubation. Fifty clutches (150 eggs) received 1cc/egg surface application of North Slope Crude Oil and 25 clutches (75 eggs) received the identical doses of mineral oil. Both treatments were delivered by drops from calibrated syringes. The initial dose was selected to be well below the lethal level of oiling for adult waterfowl (7.0-3.5 gms) reported by Hartung (1963), but is nearly completed lethal for eggs. Most evidence of petroleum exposure disappeared the next day except for slight petroleum odor (Figure 4).

Observed clutch size in the oiling experimental area initially declined at a rate compatible with normal predation from other gulls (Figure 5), but in July egg loss accelerated due to adult gulls abandoning unhatched clutches after incubation prolonged 100% longer than normal. At this time we terminated the experiment. A month after hatching began in the adjacent control colony, 33% of eggs oiled with North Slope Crude and 24.4% of eggs to which mineral oil had been applied remained in the nests on 15 July. These figures can be compared to 2% of eggs in the adjacent control area remaining in nests at the end of incubation, a 'normal' infertility rate (Figures 6, 7).

Petroleum Exposure Results

Table 2

Nests 1 - 10 Time: Onset of Incubation

Dose: 20µl	30 eggs	Date: 24 May
9/30 hatch	=	30%
6/30 "lost" (predated)	=	20%
15/30 failed to hatch	=	50%

Table 3

Nests 11 - 30 8th day of Incubation

Dose: 50µl	60 eggs	Date: 1 June
16/60 hatch	=	27%
14/60 "lost"	=	23%
30/60 failed to hatch	=	50%
early (1-2 days) chick mortality 3/60 eggs	=	5%

Table 4

Nests 31 - 50 11th day of Incubation

Dose: 100µl	60 eggs	Date: 4 June
41/60 hatch	=	68.3%
11/60 "lost"	=	18.3%
8/60 failed to hatch	=	13.3%
early (1-2 days) chick mortality 2/60 eggs	=	3%

Table 5

Mineral Oil Experiment

7th day of incubation

Nest #	# Eggs	Dose in µl	Failed to Hatch (Eggs Remaining)	Chick Mortality (Known)
51	3	20	0	
52	3	20	0	
53	3	20	0	
54	2	20	0	
55	3	20	0	
56	3	50	0	
57	3	50	0	
58	3	50	0	
59	2	50	0	
60	2	50	0	
61	3	100	0	
62	3	100	0	
63	2	100	0	
64	2	100	0	
65	3	100	1 died pipping	1 dead chick (5 days)
15 nests	40 eggs	3 different doses	mortality not significant	

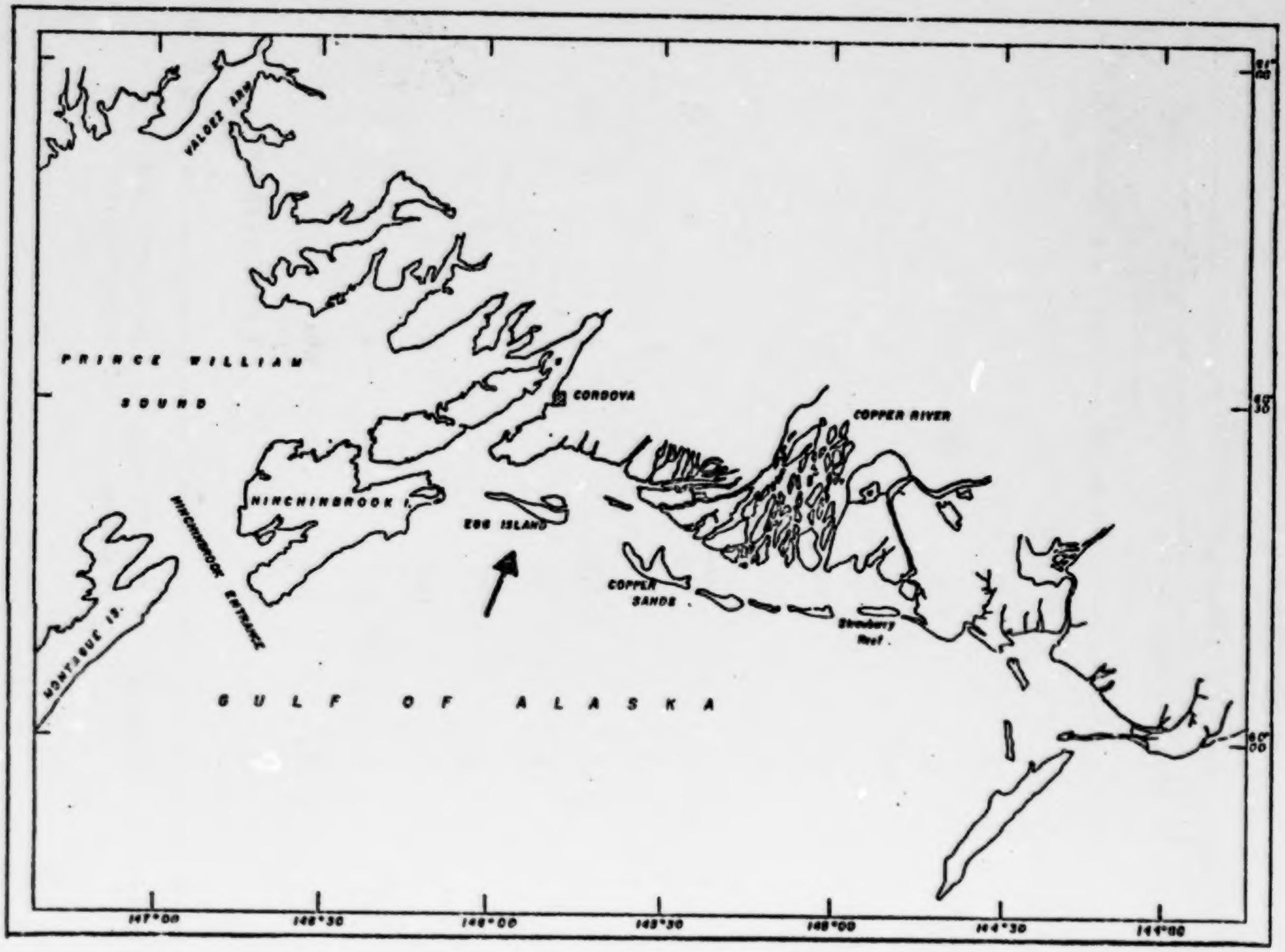

Figure 1. Map of the Copper River Delta region and Prince William Sound, showing location of Cordova, the Copper River, Egg Island (arrow), Copper Sands, and Strawberry Reef. Tanker traffic passes through Hinchinbrook Entrance enroute from Valdez.

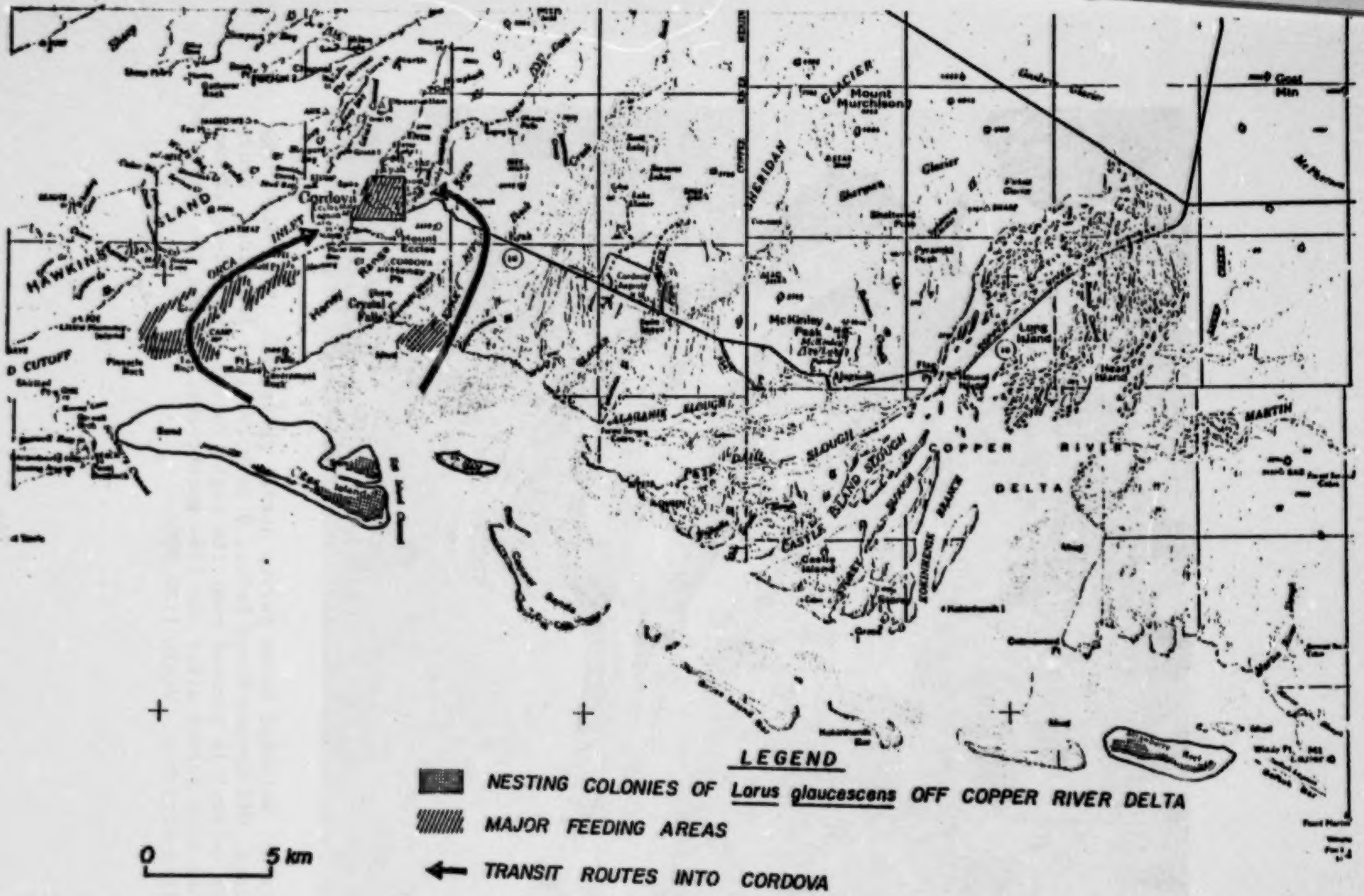

Figure 2. Map of nesting colonies off Copper River Delta, major feeding areas, and transit routes into Cordova.

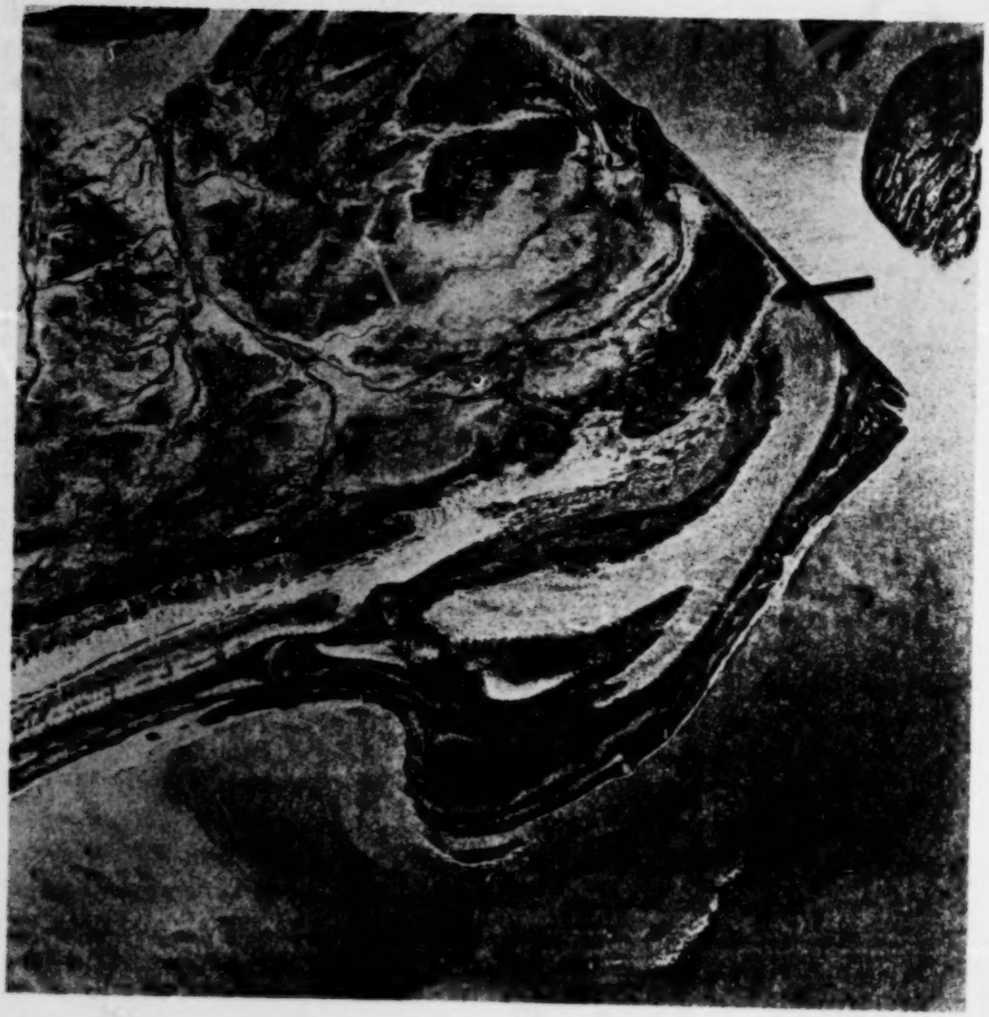

Figure 3. National Ocean Survey aerial photograph of E end of Egg Island, Off Copper River Delta, 9 July 1971, at low tide. Study Area (arrow) is located near the Light Tower. New ridges of sand dunes have formed after the 1964 earthquake, joining the series of islets together. Scale 1:30,000.

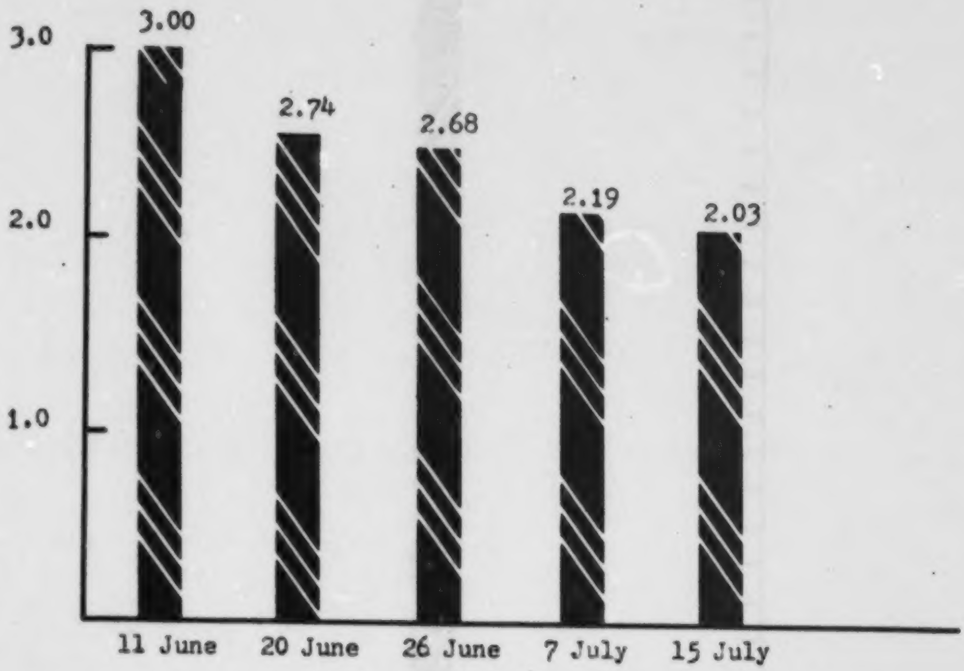

Figure 4. Observed clutch size in the oiling experimental area initially declined at a rate compatible with normal predation from other gulls.

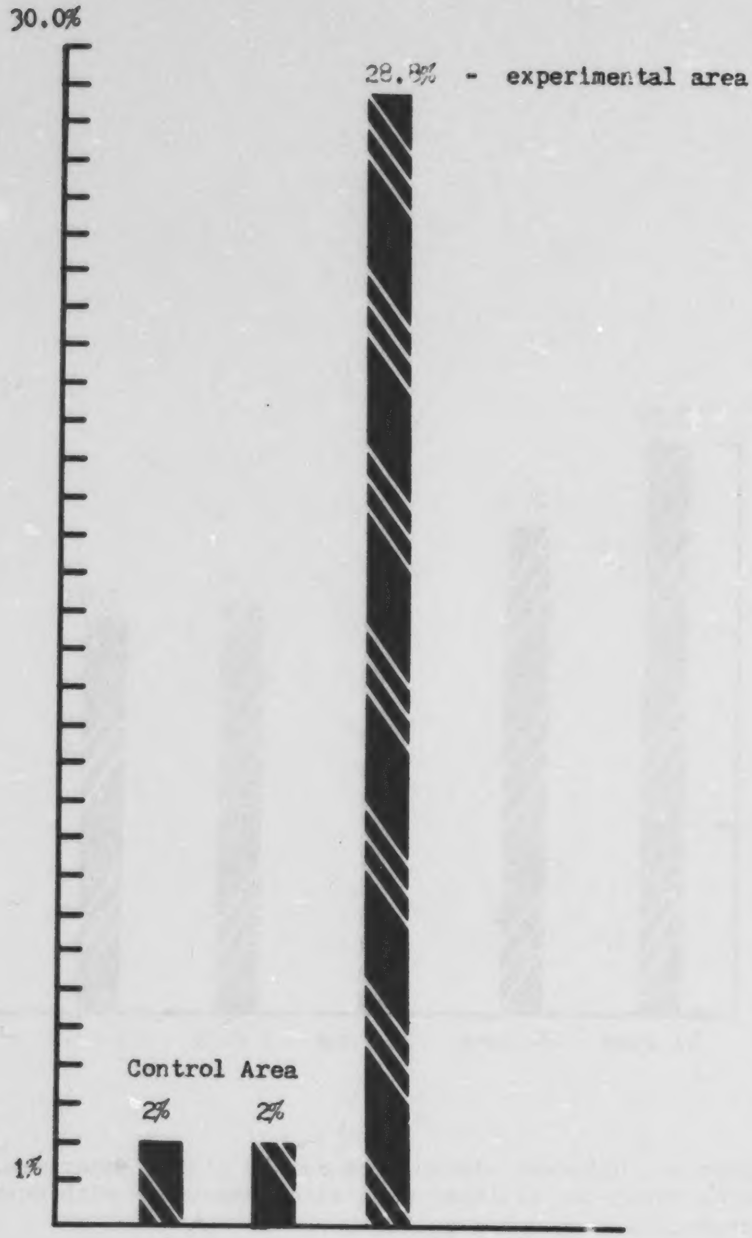

Figure 5. Precentage of eggs remaining in nests at close of incubation or experimental period, experimental and control colonies, Egg Island, 1975-1976.

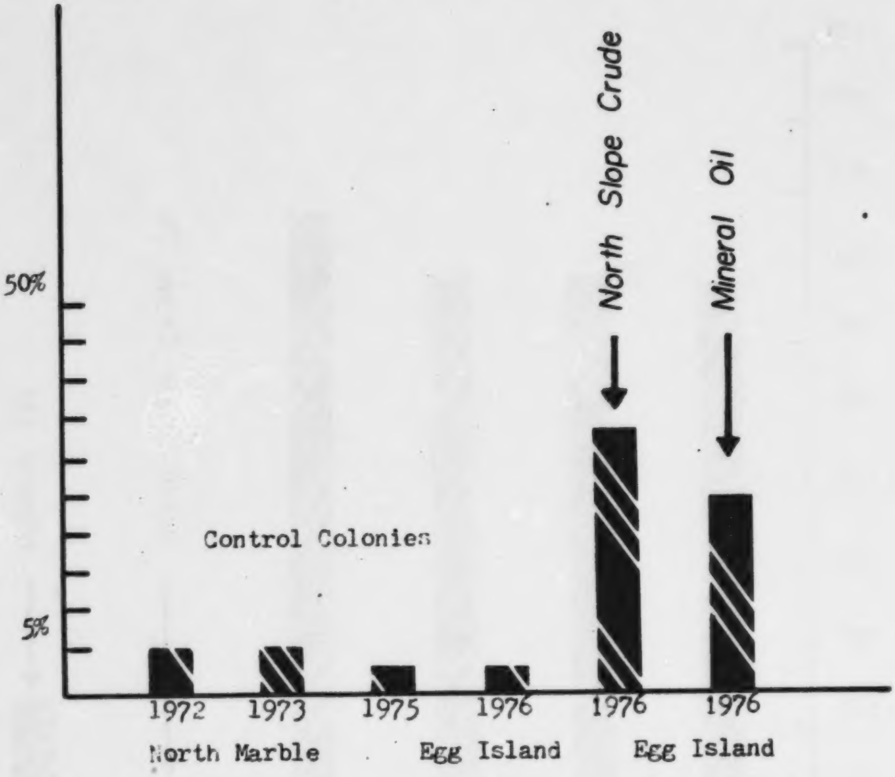

Figure 6. Percentage of eggs remaining in nests one month after hatching begins.

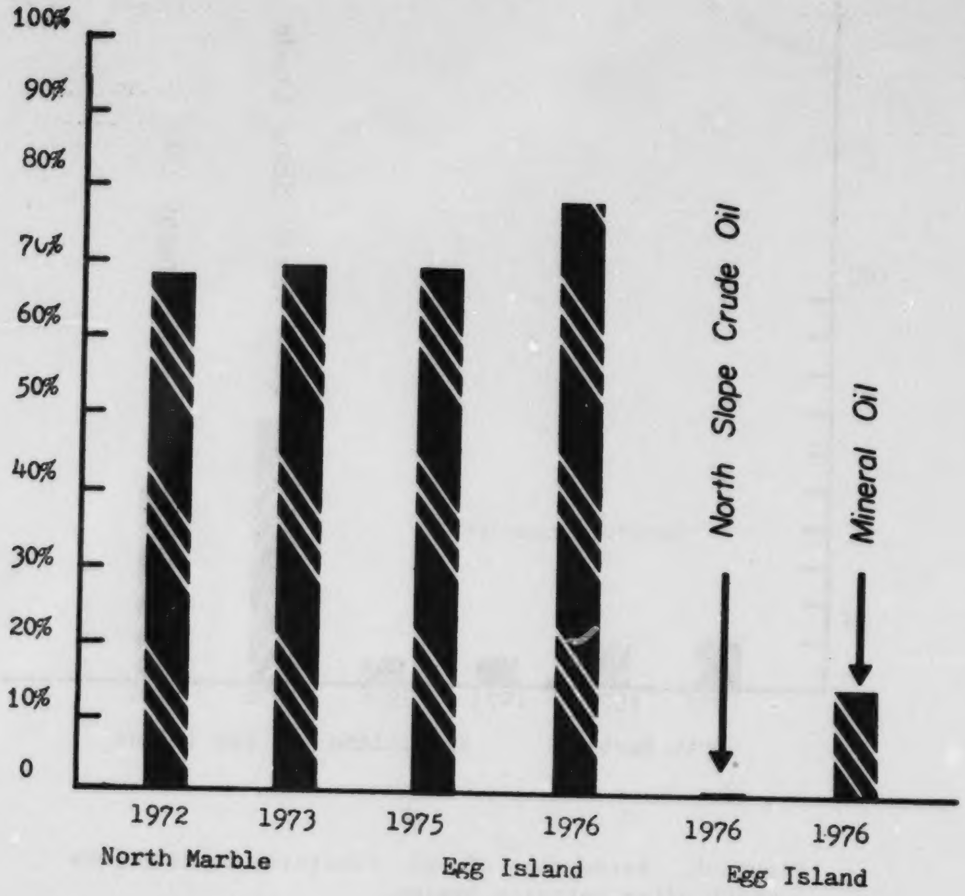

Figure 7. Hatching success.

Hatching success in eggs exposed to this nearly completely lethal dose of North Slope Crude was 0.67% (Figure 8). Mineral oil applied in equivalent amounts to gull eggs led to a hatching rate of 14.6%. North Slope Crude Oil is thus calculated to be 22 times more toxic than equivalent amounts of mineral oil. Hatching success in the adjacent 'normal' control colony was 77%; the normal rate for these gulls in Alaska is 67%-77%. Adults continued to incubate almost all unhatched clutches at least 20 days longer than normal. Eggs opened at the close of the experiment were highly decomposed and no living embryos were found. Adult gulls nesting in the oiling area produced no more replacement clutches than the neighboring control colony (4.0% vs. 4.8%) (Figures 9, 10).

Gull behavior is thus altered by the continued incubation of dead eggs. Adults fail to respond with the normal production of replacement clutches, which normally follow clutch loss to natural causes. The combination of high egg mortality and alteration of adult behavior virtually eliminated gull reproduction in the experimentally oiled area.

PROBLEMS ENCOUNTERED

No major problems encountered.

RECOMMENDATIONS FOR FUTURE RESEARCH

The literature on effects of petroleum exposure to marine bird reproduction is highly limited, and the field is completely open for continued experimentation. Existing studies demonstrate marine bird eggs are highly vulnerable to minute amounts of petroleum, several orders of magnitude less than necessary to cause adult mortality. Egg mortality is a subtle variable, and not as easily observed as adult seabird mortality. Potential effects on marine bird reproduction would not be observed until the adult population 'crashed' after some years of failure to reproduce.

Potential effects of petroleum exposure to seabird eggs hinge on transfer of petroleum during egg-laying or incubation. We are devising a protocol for capture of breeding adults, with subsequent artificial oiling, to test transfer to eggs. Necessary levels of contaminants needed to be established first, which we have now provided.

We are currently compiling information on petroleum effects on gull chick fledging success. We will carry out an expanded study of petroleum effects on gull chick viability during the 1978 field season.

Demonstrated effects to date have involved the use of unweathered North Slope Crude Oil. Petroleum encountered in the environment may or may not be weathered. The effects of weathered petroleum on marine bird

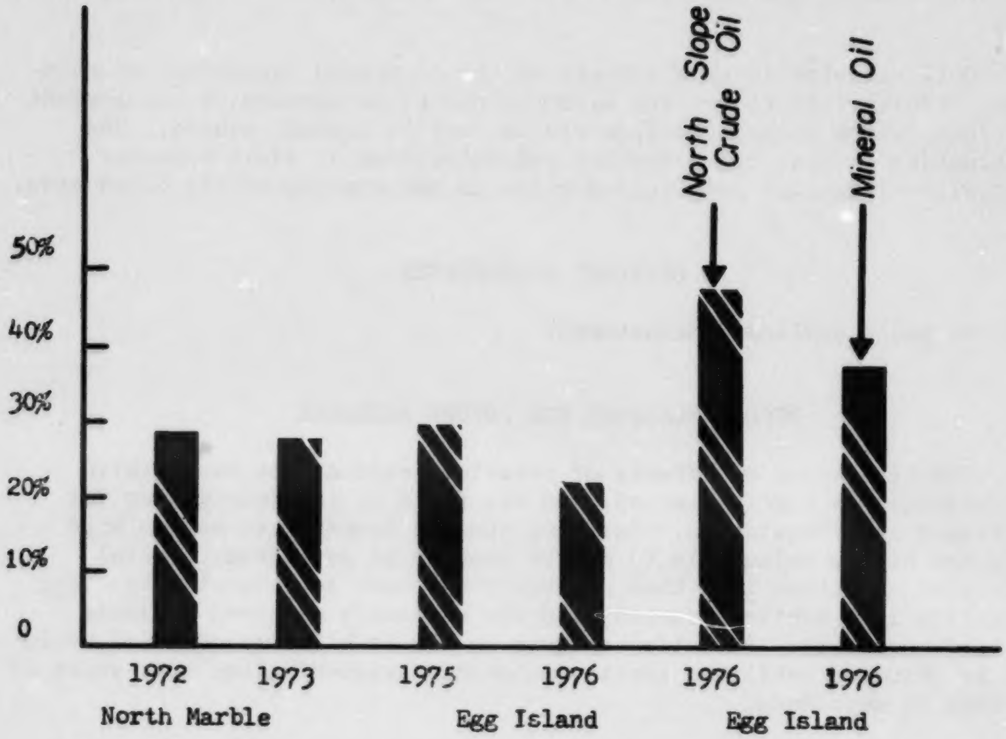

Figure 8. Known egg loss.

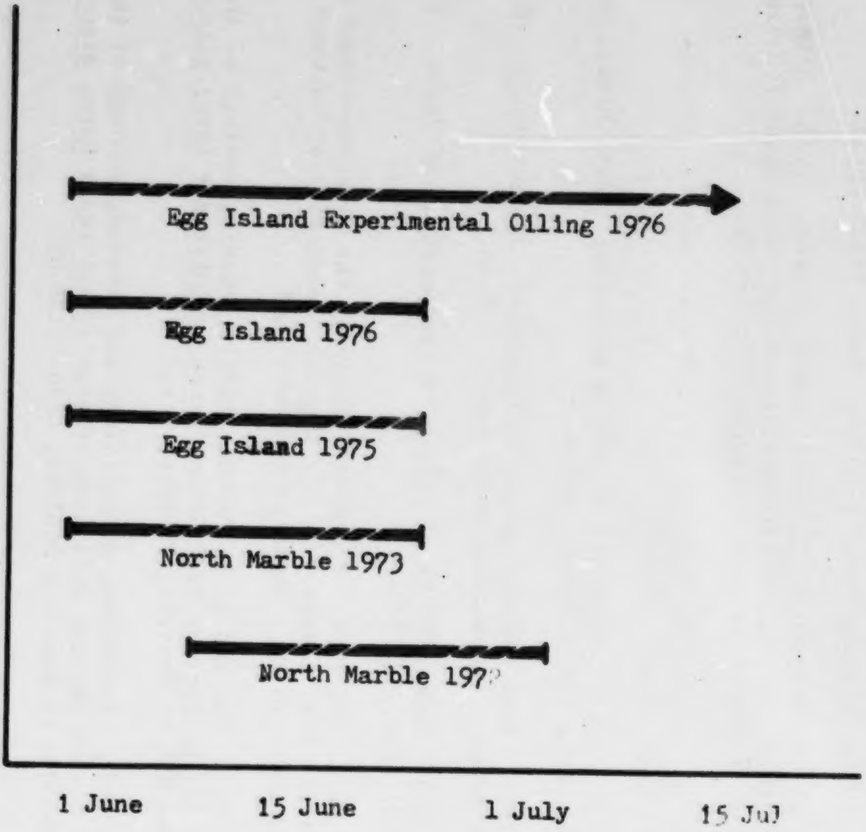

Figure 9. Median incubation periods, experimental and control colonies.

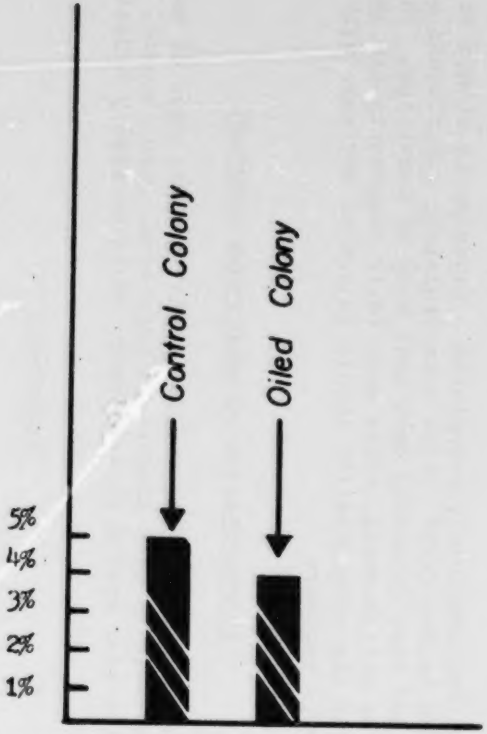

Figure 10. Percentage replacement clutches, experimental and control colonies.

reproduction are completely unexplored. Experiments should be designed and conducted in the field, first recapitulating experiments using 'raw' petroleum, and then expanding upon that body of knowledge. The comparison should be enlightening, since most toxic components are apparently volatile, and the concentration should expect to decrease with duration of atmospheric exposure.

RECOMMENDATION TO REGULATORY AGENCIES

Our information to date indicates that because eggs of marine birds are so vulnerable to tiny amounts of petroleum, even chronic, low-level oil pollution near marine bird colonies would be highly undesirable.

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11. EFFECTS OF OILING ON TEMPERATURE REGULATION IN SEA OTTERS

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BACKGROUND

This project represents an extension of studies that began with the investigations of diving and feeding behavior, and thermo-regulatory effects of oil pollution in fur seals. In this species which relies on fur for insulation against cold sea water, the effects of oiling were profound. It was thought, but not measured, that sea otters are even more dependent on their fur for insulation. The results will provide an answer to such questions as well as help in providing basic information on the general aspects of respiration in all marine mammals. Furthermore, determination of the characteristics of diving, and measurements of the energetics involved will help to provide general information on the energy requirements of the various marine mammals and their different modes of propulsion.

OBJECTIVES

1. Energy requirements of normal sea otters at various water temperatures.
2. Energy requirements of sea otters after oiling.
3. Appropriate procedures for rehabilitating oiled sea otters.
4. At-sea behavior and energetics of sea otters.

These objectives will provide a data base from which the assessment of any kind of oil contamination, or other activity which may alter the nature of the otter's food sources can be derived. In addition, relative to oil contamination the difficulties and costs of protecting from oiling, and the rehabilitating of oiled otters can be estimated.

It is predicted that oil will have an impact on sea otters through increasing their maintenance costs due to increased heat loss in air and in water as a result of pelage contamination. The magnitude of this increase will also vary between neonates and adults. External oil may also impair their diving and feeding abilities. Kenyon (1974) noted that malnutrition was common in contaminated fur seals. Finally we would anticipate direct metabolic effects of ingested oil. This proposed research will continue physiological research on sea otters, and will include an analysis and evaluation of other studies on the effects of oil pollution on sea otters.

MATERIALS AND METHODS

Two otters are presently being studied at the Physiological Research Laboratory, Scripps Institution of Oceanography. After conditioning the otters accepted confinement in the metabolic test chamber, and their normal metabolic rates were measured at a variety of water temperatures. The otters will be oiled by brushing a liberal amount of oil on their dorsal surface. Metabolic measurements will be repeated several hours after their pelts are oil-fouled. In the interim they will be allowed to swim in a small tank to remove excess oil and volatiles. At this time Dr. Sam McGinnis will conduct some of the heat flux measurements which are pertinent to an independent project. Immediately thereafter the otters will be anesthetized (for ease of handling; safety, and thoroughness) and their fur will be cleaned of oil with a detergent. After 1 or 2 days recovery from anesthesia, maintenance metabolic rates will again be measured at intervals for 2 or 3 more weeks. Data will be analyzed to show changes in metabolic rates associated with alteration due to oil of the insulative properties of the fur, and changes associated with the cleansing process. Results will be compared with data from past studies on northern fur seals, and with the results of other investigators on these and other species.

The sampling procedures will be the same as those recently used for fur seals and used previously in metabolic rates in penguins (Kooyman, G. L., R. L. Gentry, W. P. Bergman and H. T. Hammel, 1976, Comp. Biochem. Physiol. 54A: 75-80).

The thermal neutral zone will be determined in four sea otters conditioned to "rest" in the metabolic test chamber. The principle variable measured in these tests is oxygen consumed, and body and skin temperature. The control thermal neutral zone will be compared to otters after oiling and after cleaning. Furthermore, the continuous sampling ability of our method will permit us to determine the average whole body heat conductance for a 10 to 12 hr run. This will include the important activity (mainly grooming) periods. The changes in whole body conductance during exposure to various water temperatures before and after oiling will indicate the metabolic costs of oil on the fur. These same sampling procedures will be repeated after the oiled animals have been anesthetized and cleaned.

RESULTS

To date 38 six hour metabolic runs at six different water temperatures between 5 and 30°C have been made. The thermal neutral zone for the otters has been preliminarily established and appears to have a lower critical temperature between 5 and 10°C (figure 1). The metabolic rates recorded are in excellent agreement with other published estimates (Morrison, Rosenmann, and Estes 1975; Iverson and Krog, 1973). Prudhoe crude oil has been applied to one otter on two different occasions and changes in metabolic rate after oiling and washing have been made. The application of 38 mls and 60 mls of crude oil upon the dorsal surface of the sea otter increased the otters average metabolic rate 24%, and 60% respectively.

Upon washing the metabolic rate increased 115% and 53 % above normal for the two oilings respectively (figure 2). The otters metabolic rate had returned to normal by the eighth day for the first oiling and was still slightly elevated by the 14th day for the second oiling. Upon washing, the fur of the entire animal appeared wet and the otter was observed shivering. This was a response to the increased heat loss due to the loss of insulating quality of the fur after washing. After the first oiling the washed otter recovered in our normal holding facility. However, after the second oiling, the washed otter was not capable of completely grooming her fur into condition. Forty-eight hours after washing she had only groomed her upper torso and the fur on her lower abdomen was totally wetted. During this time the otter shivered constantly. The lack of ability to groom and the shivering was probably a result of the coooler sea water temperature in the holding tank between the two oiling experiments. During the first oiling, the holding tank water temperature was 5°C higher than during the second oiling. Due to the inability of the ottter to groom itself properly and its constant shivering the otter was removed from the holding tank and placed in a small water tub filled with 25-30°C water. The tank was left filled for three hours and then drained and left empty for 1-2 hours and then filled again. This procedure was carried out for 24 hours. After the 24 hour period the otter had managed to successfully groom her entire body and was returned to our regular holding tank where she continued to groom normally and was not observed to shiver again.

However, two weeks after the oiling experiment, routine blood samples were taken from both otters for routine examination and the otter which had been oiled exhibited an unusually high white blood cell count. The otter was then transferred to Sea World for veterinary care and is currently still at Sea World suffering from what they believe to be a deep seated pneumonia. The pneumonia is probably a result of the stress encountered by the otter during the post-washing period.

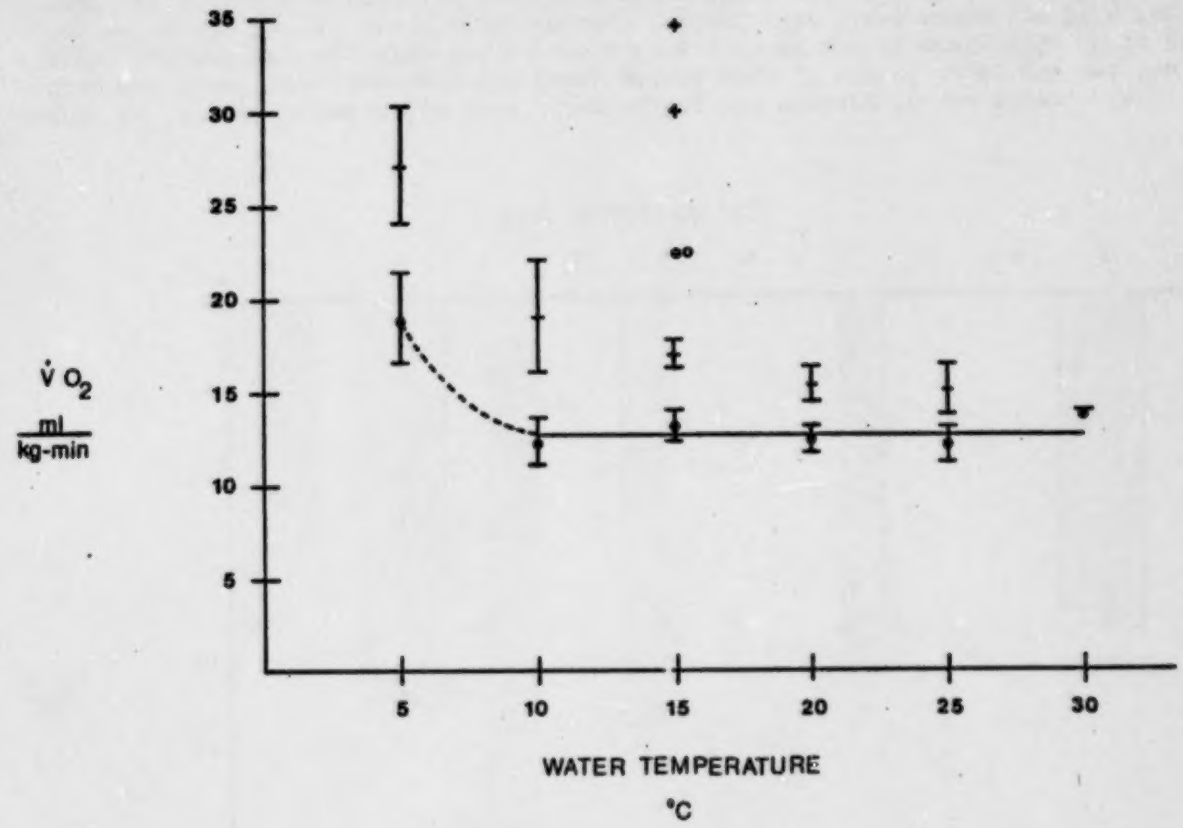

Figure 1. Relationship between water temperature and metabolic rate for the three otters studied. The closed dots are the mean resting metabolic rate, the lined bars, the average metabolic rate. The open dots are the resting rates for the two oilings and the + are the resting washed rates. The error bars represent one standard error for 1,6,6,10,2,4, six hour runs at 30, 25, 20, 15, 10 and 5@C respectively.

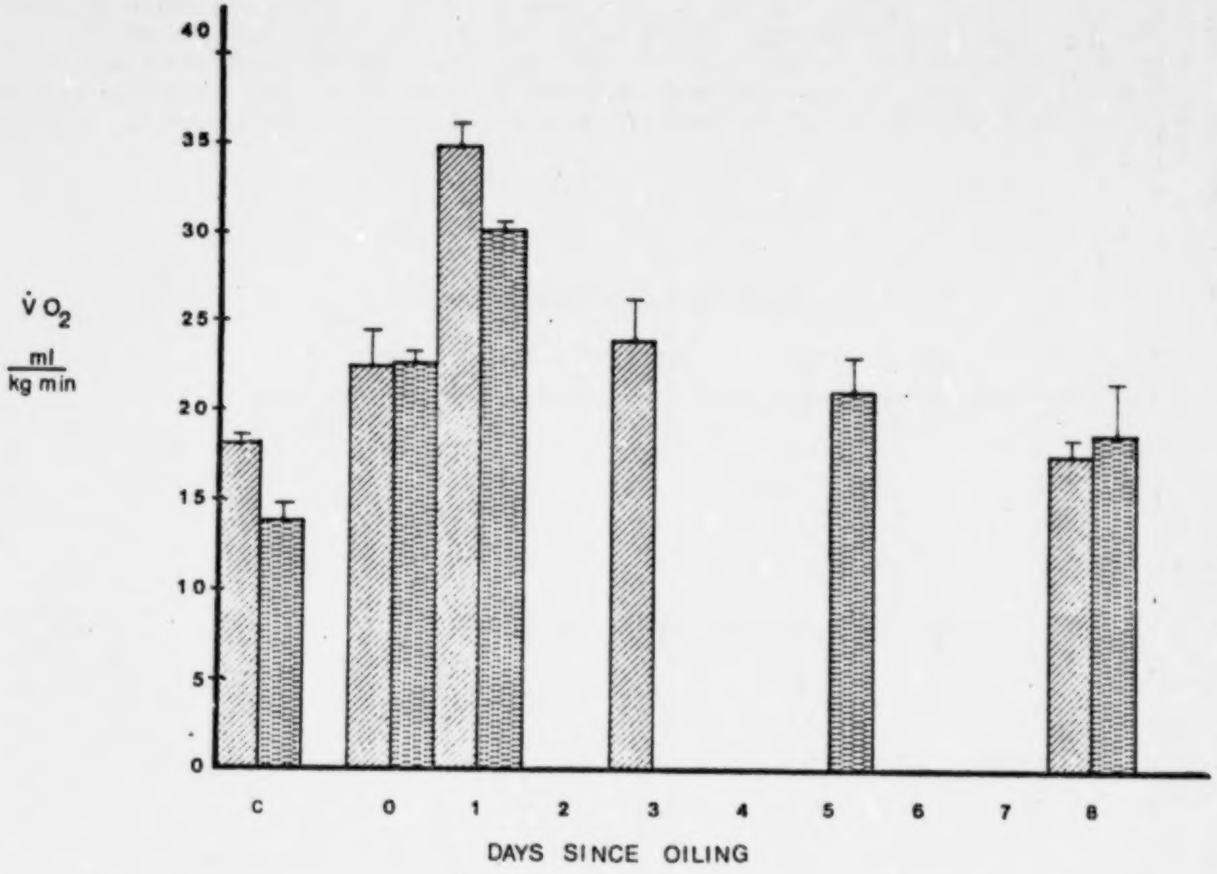

Figure 2. Change in metabolic rate after oiling and washing in one otter. The diagonal lined bars represent the first oiling with 38 mls of crude oil and the cross hatched bars are from the second oiling with 60 mls of crude oil. C is the control value for the otter prior to each oiling. The lines above the bars represent one standard error. The number of measurements per 6 hour run was 5 for the first oiling and 6 for the second.

FUTURE RESEARCH

In order to assess the effects of oil on otters in the wild we are planning to attach a frequency and depth of dive recorder on control and oiled sea otters in Prince William Sound, Alaska. From these data we will be able to quantify the changes in sea otter foraging and diving due to oil contamination. In addition we are planning to enlist the help of Dr. Richard Harrison in studying the skin glands in sea otters so that we might be able to identify adaptations which allow the fur to retain an air layer. In addition determination of the composition of the natural fur oils would be beneficial in creating an artificial oil which could be used to replace the natural oils after washing.

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PART II

STUDIES FUNDED UNDER FEDERAL INTERAGENCY ENERGY/ENVIRONMENT PROGRAM THROUGH THE ENVIRONMENTAL PROTECTION AGENCY AND THE NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION

12. AN OVERVIEW OF NOAA'S ENERGY RESOURCES PROJECT ON FATE AND EFFECTS OF CONTAMINANTS IN MARINE ECOSYSTEMS AND ORGANISMS

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In 1975, the federal government initiated the Interagency Energy/Environment R&D Program, which is administered and coordinated by EPA's Office of Energy, Minerals, and Industry. Research and development activities under this program are performed by more than a dozen federal agencies, including NOAA. The overall interagency effort is subdivided into two major parts:

- R&D on effects of pollutants, including health effects, ecological effects, pollutant transport (dissemination) and fate, and integrated assessment of all type of effects. This effort is termed the Processes and Effects Program.
- R&D on technological measures for controlling the release of pollutants to the environment, including changes within an energy extraction, conversion or utilization process as well as end-of-system control measures. This effort is called the Control Technology Program.

NOAA administers several projects under the Processes and Effects Program including the Fate and Effects Project under discussion here and environmental assessment efforts in the northern Puget Sound and in the Buccaneer Oil Field of the Gulf of Mexico. Additional information on these and related aspects of the overall Interagency R&D Program has been summarized by EPA (1977a,b).

The general objectives of the National Oceanic and Atmospheric Administration's (NOAA) energy resources project on fate and effects are to identify and conduct laboratory and field research studies that will facilitate the assessment of potential environmental impacts from petroleum releases in coastal marine ecosystems. This effort does not have a site-specific orientation; instead, it is focused on the fundamental processes that control the distribution, transport, and effects (including physiological and ecological aspects) of petroleum components, toxic metals, and other contaminants in coastal ecosystems. It is expected that the results of the process-oriented studies conducted under this project will have broad applicability to environmental problems in widely separated geographic areas.

The technical development plan (NOAA 1975) for the energy resources project on fate and effects identified four major tasks to be undertaken:

1. Establishment of a NOAA national analytical facility for analysis of petroleum hydrocarbons and toxic metals in the marine environment.

2. Synthesis of existing information and identification of research needs on fate and effects of toxic metals and petroleum hydrocarbons in subarctic marine ecosystems.

3. Laboratory research on specific effects of transport processes involving petroleum hydrocarbons and toxic metals in marine ecosystems.

4. Experimental ecosystem research on ecological responses to additions of petroleum hydrocarbons.

During the first year, the efforts of this project on fate and effects were oriented mainly toward establishment of suitable in-house analytical support capability for NOAA's overall environmental program and the planning for future studies on fate and effects, objectives 1 and 2, above. As part of this planning effort, a major symposium was conducted on November 10-12, 1976, under the title "Fate and Effects of Petroleum Hydrocarbons in Marine Ecosystems and Organisms." The symposium, which was attended by approximately 350 persons from several countries, was intended to provide a vehicle for documenting the current status of research on the fates and effects of petroleum in the marine environment and for identifying areas most in need of future research. Twelve recognized experts were invited to present summary reviews on various aspects of petroleum in the marine environment. In addition, to provide a detailed status of current research, contributed papers were solicited from the research community-at-large on this topic. Seventy-one abstracts were received in response to this solicitation, and 58 of these were selected by the symposium planning and editorial committee for actual presentation. Fifty-one manuscripts were submitted for publication in the symposium proceedings, and 35 of these were accepted by the committee. The final proceedings volume (Wolfe, 1977a) includes the invited and the accepted contributed papers, as well as the panel discussion, which took place on the last day of the symposium. During this discussion, the panel and the audience expressed their thoughts on where the research priorities of the future ought to lie. These recommendations have been summarized previously (Wolfe, 1977b).

Several subprojects have been initiated to address objectives 3 and 4 of NOAA's Fate and Effects Project, and these are described by the principal investigators in the subsequent papers of this review proceedings. The realities of ecosystem complexity and natural variability severely restrict the utility of controlled field experiments and demand a high degree of planning and experimental design prior to implementation. As a first step toward the necessary planning, NOAA (through Resource Management Associates) is conducting design feasibility analyses for various experimental approaches and ecological hypotheses that might be appropriately tested in field perturbation experiments with petroleum. Results of the study are summarized in the last paper of this section, and will soon be available as a full report.

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13. THE NOAA NATIONAL ANALYTICAL FACILITY -- A STATUS REPORT

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The NOAA National Analytical Facility was established at the Northwest and Alaska Fisheries Center to provide NOAA's environmental programs with detailed information on the types and amounts of trace chemical contaminants in the marine environment. Many of NOAA's environmental programs, such as those concerned with problems related to spilled oil, must contend with extremely complex mixtures of chemical compounds which are challenging to isolate, identify, and measure. In order to deal effectively with such materials, highly trained analytical chemists and technicians are needed to operate the modern, sophisticated instrumentation required for these trace chamical analyses. This presents difficulties for many research projects which have neither the resources nor the volume of analyses to justify such an undertaking, yet quality analytical data are required to accomplish the research objectives. Foreseeing these analytical requirements, NOAA's Energy Resources Project III and NOAA's National Marine Fisheries Services (NMFS) jointly sponsored the founding of the National Analytical Facility.

ORGANIZATION

The National Analytical Facility has a permanent staff of six chemists, augmented by six nonpermanent chemists and technicians, and approximately 12 student-chemists and aides. Organizationally, the NOAA National Analytical Facility is part of NMFS, located within the Environmental Conservation Division at the Northwest and Alaska Fisheries Center. At this research center, the Facility is associated with an experienced multidisciplinary team of chemists, biochemists, biophysicists, and physiologists engaged in studies on contaminant-induced changes in marine biota. Interaction with this team of scientists is important in relating the analytical work of the Facility to environmental issues and placing interpretations of the data in an environmental perspective.

MISSION

In its associations with research groups at the Center, elsewhere in NOAA, and with other Federal and state agencies, the Facility serves as a focal point for standardizing analytical methodology, certifying the assurance of quality of the cooperating analytical laboratories, and providing information on the validity, sensitivity, and precision of analytical data. The Facility was planned to undertake six primary functions:

Recommend analytical procedures needed to accomplish the objectives of NOAA's environmental programs.

Perform state-of-the-art analyses on complex extracts from marine water, biota, and sediment in substantial numbers, as needed.

Adapt and develop analytical technology essential to NOAA programs.

Interpret analytical data in relation to the affected biota, and place this information in perspective for administrative decision and actions.

Provide contract liaison and quality assurance when analyses are performed for NOAA projects by other laboratories.

Conduct or collaborate in intercalibratiion studies on detecting, identifying, and measuring trace contaminants in environmental samples to ensure that analytical results can be compared among various studies.

INITIAL FUNDING

The NOAA National Analytical Facility was launched during 1976 with $310,000 in EPA Pass-Through funds as part of the Federal Interagency Energy/Environment Research and Development Program. These EPA funds made possible the acquisition of highly sensitive and discriminating analytical instrumentation. The first requirement was the combined gas chromatograph/mass spectrometer equipped with automation to record and process substantial quantities of analytical data. The cost of this instrumentation exceeded $130,000. Next, two automated high resolution gas chromatographs were purchased to process most of the routine trace contaminant meaurements. To analyze for some of the more toxic or potentially carcinogenic constituents of petroleum and their metabolites, the Facility acquired two high pressure liquid chromatographs and an ultraviolet fluoresoence spectrometer. The least expensive of these last five instruments cost $16,000. This equipment, in combination with exacting sample workup procedures, enables skilled and experienced Analytical Facility chemists to detect, identify, and measure individual trace chemical contaminants in marine sediments, biota, or seawater down to parts-per-billion levels. The functions of these instruments are listed in Table 1.

In addition to equipping the Facility, the EPA funds supported preliminary studies essential to determine the best procedures for analyzing oil in the environment. The EPA funds also enabled the staff to be trained in various specialized techniques. This support helped ensure that EPA-funded NOAA Energy Resources Projects benefited from state-of-the-art analytical services. For example, the Facility performed $17,000 worth of chemical analyses for the NOAA/EPA investigation, "Effects of Chlorinated Hydrocarbon Residues on the Bioaccumulation and Toxicity of the Petroleum Hydrocarbons" performed by the Environmental Conservation Division at this Center. The Facility also managed quality assurance analyses for the NOAA/EPA Buccaneer Oil Field Study by the NMFS Galveston Laboratory. In NOAA's Outer Continental Shelf Environmental Assessment Program (OCSEAP), funded by the Bureau of Land Management (BLM), the Facility provides technical consultation and trace organic chemical analyses for environmental projects in NMFS laboratories at Seattle WA, Auke Bay AK, and Tiburon CA. During fiscal year (FY) 1977, the Facility provided $60,000 in analytical services to these three OCSEAP projects.

MAJOR PROJECTS

In addition to providing chemical consultation and analytical services to principal investigators on NOAA research projects, the Facility is associated with several research projects in which it is the principal investigator. Major projects are summarized in Table 2. Detailed descriptions follow.

NORTHERN PUGET SOUND HYDROCARBON BASELINE

The initial EPA funding complemented NOAA's Energy Resources Project I in its EPA funded studies on the potential impact of petroleum on the marine environs of Northern Puget Sound. This inland marine region has been largely free of oil contamination, but recent sharp increases in tanker traffic have necessitated documentation of existing baseline levels of hydrocarbons to assist in assessing the impact of future oil contamination. Thus, the NOAA-managed EPA funds helped equip, staff, and train an advanced analytical facility which could then conduct EPA funded studies on essential hydrocarbon baseline data for an important inland marine area.

The Puget Sound Project of NOAA's Marine Ecosystems Analysis (MESA) Program provided $75,000 in EPA Pass-Through funds to conduct a pilot study to address issues such as the types of petroleum constituents to be documented; the numbers and kinds of samples to be collected for

TABLE 1

INSTRUMENTATION	APPLICATION
GAS CHROMATOGRAPH/MASS SPECTROMETER	
Two units, with computer, high resolution, chromatographic columns and choice of ionization (EI/CI).	Used to identify unknown organic compounds at trace levels in complex extracts, and to confirm the identity of compounds determined by routine high resolution gas chromatography, high pressure liquid chromatography or ultraviolet fluorescence spectrometry.
GAS CHROMATOGRAPH	
Three units, microprocessor controlled for automatic sample injection and data processing, with high resolution columns (glass capillaries) and choice of three detectors.	Used to separate complex mixtures into their constituent compounds for measurement at parts-per-billion levels; automation permits 24-hr operation; requires alternative techniques to confirm the identity of compounds detected and measured.
HIGH PRESSURE LIQUID CHROMATOGRAPH	
Two units equipped for automatic data processing, one unit with automatic sample injection and choice of two detectors.	Use is analogous to the gas chromatograph; finds best application for analyzing compounds not well suited for gas chromatographic analysis due to instability or low volatility.
ULTRAVIOLET FLUORESCENCE SPECTROMETER	
Dual wavelength synchronous scanning capability, equipped with a microprocessor to record, store, and correct fluorescence spectra.	Used to detect low levels of toxic and potentially carcinogenic organic compounds in oil, and to follow metabolic transformations of these compounds; used for rapid comparison of sample extracts with suspected sources of oil contamination.

TABLE 2. NOAA National Analytical Facility - Summary of Projects

NOAA Project	Funds, $K FY 76	FY 77	FY 78	Source
Establish National Analytical Facility	235	75		NOAA/EPA
Services				
Energy Resources Project III	18	17	19	NOAA/EPA
OCSEAP Projects (3)		60	35	OCSEAP/BLM
Northern Puget Sound Hydrocarbon Baseline	75	140	ca.80	NOAA/EPA
Prince William Sound Hydrocarbon Monitoring		80	83	NMFS
New York Bight Chlorinated and Aromatic Hydrocarbons		71	105	MESA
Argo Merchant/Bouchard Oil Spills		74	27	MESA
Intercalibration Studies			20	OCSEAP/BLM
TOTALS	328	517	369 (Estimated)	

analysis; how, when, and where to collect these samples; sample storage and preparation for instrumental analysis; choice of analytical methodology and data management; and the design of a hydrocarbon baseline investigation.

The effort culminated in the NOAA/EPA 60-page Technical Memorandum ERL MESA-8.

PRINCE WILLIAM SOUND HYDROCARBON MONITORING

With $80,000 in NOAA funds for environmental monitoring, the Facility set up sampling stations for monitoring hydrocarbons in the marine environment along the inner shipping lanes of the Northern Gulf of Alaska. Periodically, the Facility analyses intertidal sediments, biota, and water in the Prince William Sound area in connection with shipment of Prudhoe Bay crude oil from Port Valdez. The Facility collaborates with NMFS's Auke Bay Laboratory in the field sampling base. The U.S. Fish and Wildlife Service supports the Auke Bay Laboratory's costs for sampling logistics with $12,000 obtained from the Alyeska Pipeline Company. As in Northern Puget Sound, little oil contamination was evident at most stations.

NEW YORK BIGHT AROMATIC AND CHLORINATED HYDROCARBONS

The Facility is conducting a three-year, $250,000 study of the levels of aromatic hydrocarbons, chlorinated hydrocarbons and other organic chemical contaminants in the New York Bight area. Samples of marine biota, water surface microlayer, surface water, sediments, sewage sludge, dredge spoils, and free-floating fish eggs will be analyzed. This study is sponsored by the MESA/NY Bight Project. The Facility collaborates with the Milford CT NMFS Laboratory, which collects the samples in the field and forwards them to Seattle for analysis.

ARGO MERCHANT/BOUCHARD OIL SPILLS

The winter of 1976/1977 witnessed two serious oil spills in coastal waters of Massachusetts. The facility responded to the *Argo Merchant* oil spill by performing analyses of fish samples collected by the NMFS Center at Woods Hole MA, and by analyzing bottom sediments collected by the University of Rhode Island for MESA. Fuel oil samples from the ice and water environment around the recent *Bouchard Barge* spill were similarly analyzed. These studies were supported by $75,000 through the MESA Program. The overall findings of these analytical studies have recently been reported to NOAA and EPA. Evidence of *Argo Merchant* oil contamination was detected in the stomach contents of a few fish.

OCS INTERCALIBRATION STUDIES

The Facility participates in collaborative studies organized by the National Bureau of Standards to evaluate analytical methods to measure trace levels of hydrocarbons in the marine environment for BLM. In this effort the Facility periodically analyzes test samples of oil-contaminated sediments and tissues for interlaboratory comparisons among OCS researchers. With $10,000 from OCSEAP/BLM, the Facility will provide OCSEAP and BLM OCS contractors with substantial quantities of reference intertidal sediment. Before release to OCS principal investigators, ten portions of a homogenized contaminated sediment will be analyzed at the Facility to establish sample homogeneity and statistical criteria for 30-40 petroleum hydrocarbons.

In relation to its general consulting functions, the Facility has briefed the American Society of Testing and Materials (ASTM) on the various analytical methods which were employed for the major coastal oil spills of 1977. ASTM has asked the Facility to submit its procedures for analysis of hydrocarbons for consideration as ASTM approved procedures.

ANALYTICAL OPERATIONS

As indicated in Figure 1, sample preparation and analysis procedures employed by the Facility follow a common general scheme involving solvent extraction of sediments, biota, or water with or without prior homogenization and digestion; adsorption chromatography on silica gel to isolate the saturated and aromatic hydrocarbon fractions; microbalance weighing of each fraction; automatic processing gas chromatography to separate each fraction into individual chemical compounds and measure them; and computerized gas chromatography/mass spectrometry to confirm the identities of the compounds detected and measured above.

Graphs of the gas chromatographic analyses illustrate the nature and extent of petroleum contamination found in samples. Figure 2 shows the saturated hydrocarbons from sediments taken from two similar intertidal sites along the Strait of Juan de Fuca as part of the Pilot Study. The large peaks correspond to the major alkanes separated from other hydrocarbons by the gas chromatograph. The site of the peak indicates the amount of the compound present. Clearly, the Port Angeles sediment shows a larger and more complex pattern of hydrocarbons than does the sediment from Dungeness. The former is from a contaminated harbor, the latter from a natural area. The major hydrocarbons from the Dungeness sediment amount to only a few parts-per-billion based on dry weight.

The analytical data from the Puget Sound, Prince William Sound, and New York Bight Projects is entered on punch cards, using the format (Fig. 3) developed by MESA's Puget Sound Project. This information is then transmitted to NOAA's National Oceanographic Data Center in Washington DC. Computer programs have been developed for retrieval and tabulation of information on these cards.

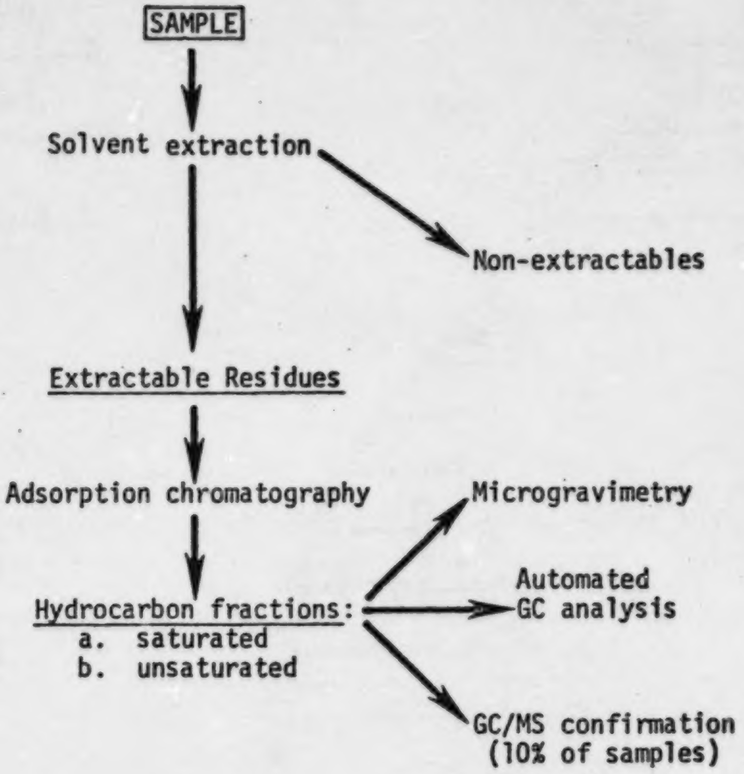

Figure 1. Sample processing scheme.

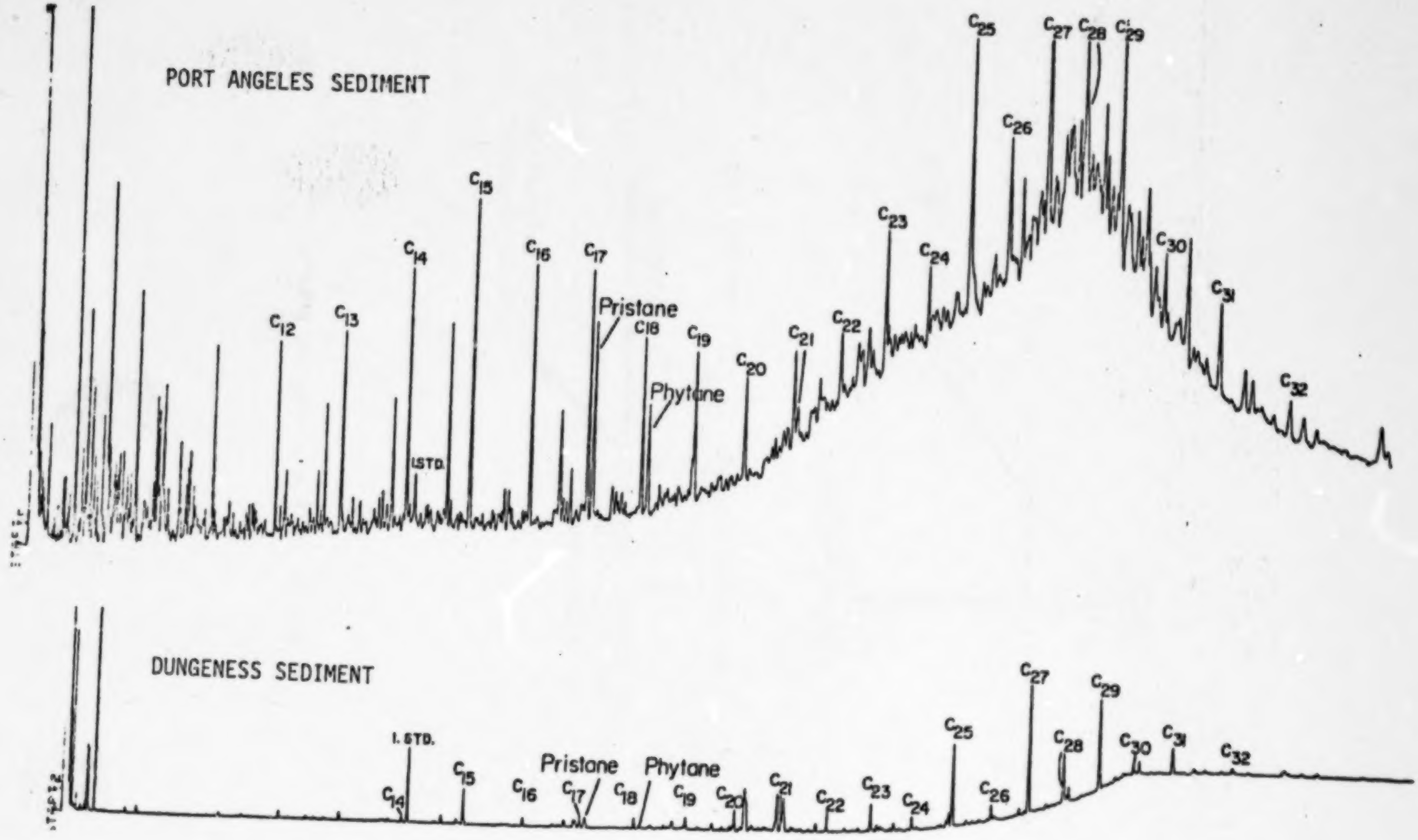

Figure 2. High resolution gas chromatograms of saturated hydrocarbons from intertidal sediments.

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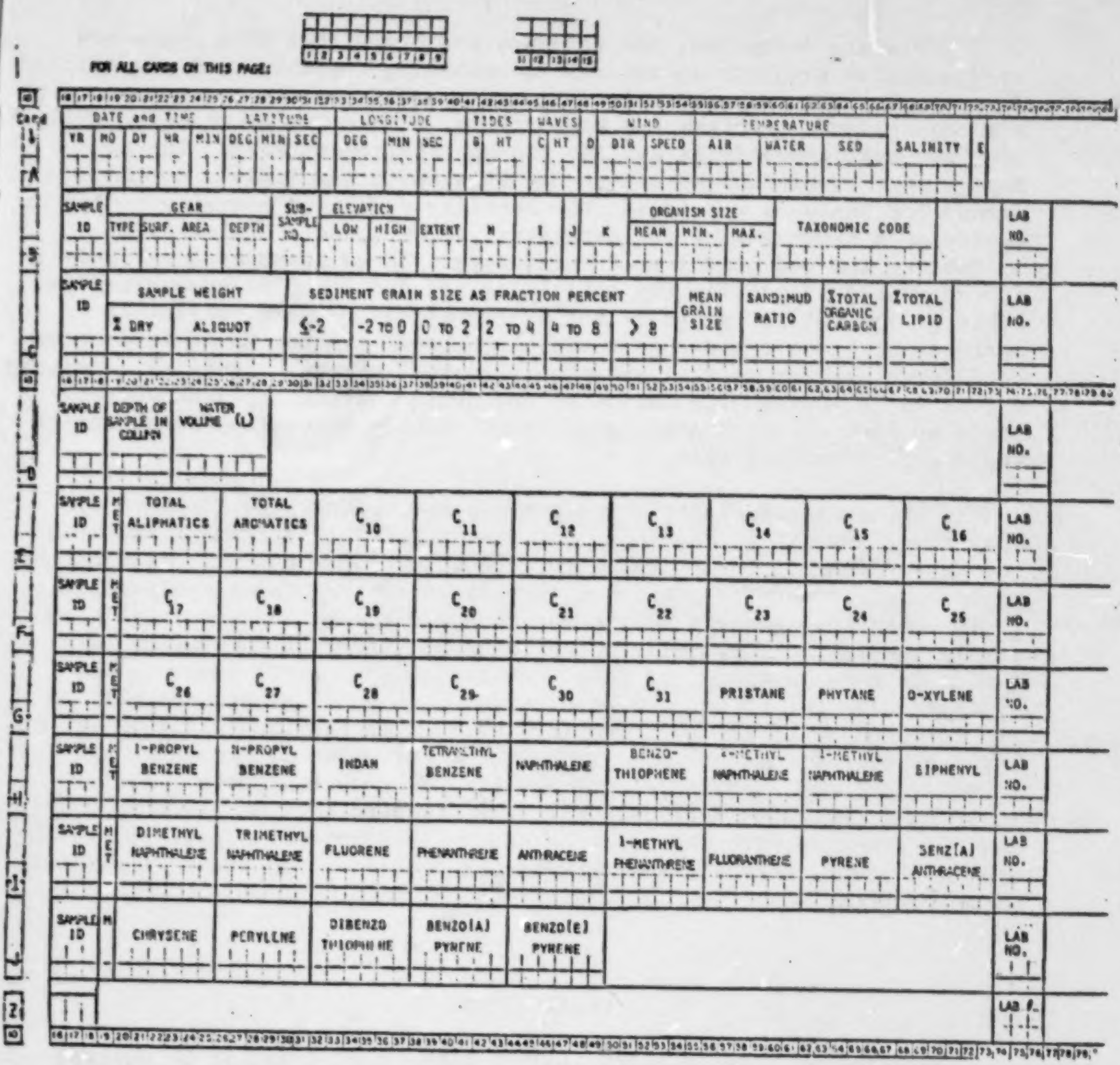

FOR ALL CARDS ON THIS PAGE:

1	2	3	4	5	6	7	8	9		11	12	13	14	15

Card A

DATE and TIME					LATITUDE			LONGITUDE			TIDES		WAVES			WIND		TEMPERATURE				
YR	MO	DY	HR	MIN	DEG	MIN	SEC	DEG	MIN	SEC	B	HT	C	HT	D	DIR	SPEED	AIR	WATER	SED	SALINITY	E

Card B

SAMPLE ID	GEAR			SUB-SAMPLE NO.	ELEVATION		EXTENT	H	I	J	K	ORGANISM SIZE			TAXONOMIC CODE	LAB NO.
	TYPE	SURF. AREA	DEPTH		LOW	HIGH						MEAN	MIN.	MAX.		

Card C

SAMPLE ID	SAMPLE WEIGHT		SEDIMENT GRAIN SIZE AS FRACTION PERCENT						MEAN GRAIN SIZE	SAND:MUD RATIO	%TOTAL ORGANIC CARBON	%TOTAL LIPID	LAB NO.
	% DRY	ALIQUOT	≤-2	-2 TO 0	0 TO 2	2 TO 4	4 TO 8	> 8					

Card D

SAMPLE ID	DEPTH OF SAMPLE IN COLUMN	WATER VOLUME (L)	LAB NO.

Card E

SAMPLE ID	MET	TOTAL ALIPHATICS	TOTAL AROMATICS	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	LAB NO.

Card F

SAMPLE ID	MET	C_{17}	C_{18}	C_{19}	C_{20}	C_{21}	C_{22}	C_{23}	C_{24}	C_{25}	LAB NO.

Card G

SAMPLE ID	MET	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	PRISTANE	PHYTANE	O-XYLENE	LAB NO.

Card H

SAMPLE ID	MET	I-PROPYL BENZENE	N-PROPYL BENZENE	INDAN	TETRAMETHYL BENZENE	NAPHTHALENE	BENZO-THIOPHENE	2-METHYL NAPHTHALENE	1-METHYL NAPHTHALENE	BIPHENYL	LAB NO.

Card I

SAMPLE ID	MET	DIMETHYL NAPHTHALENE	TRIMETHYL NAPHTHALENE	FLUORENE	PHENANTHRENE	ANTHRACENE	1-METHYL PHENANTHRENE	FLUORANTHENE	PYRENE	BENZ[A] ANTHRACENE	LAB NO.

Card J

SAMPLE ID	M	CHRYSENE	PERYLENE	DIBENZO THIOPHENE	BENZO[A] PYRENE	BENZO[E] PYRENE	LAB NO.

Card Z

	LAB #.

Figure 3. Data entry format for submission to NODC.

OUTLOOK

Since its inception, the Facility has undertaken NOAA sponsored environmental projects as rapidly as expanding capacity would permit. Accordingly, the permanent staff of six chemists has been augmented with six nonpermanent chemists and technicians. These are distributed among two four-man laboratories and one two-man instrument room. The two supervisory chemists need not occupy this space regularly. As the demand for analyses continued, the Facility sought to maximize output by employing a student-aide for each staff member, and by extensively automating the analytical instrumentation. These measures have been indispensable in meeting the activities for FY 77 and 78 summarized in Table 2. If NOAA's environmental programs require ever increasing analyses of trace organic contaminants, other measures would have to be considered such as gaining additional space, subcontracting, or accepting contracts to monitor the output of the other analytical laboratories. Since so much of the instrumentation is already automated, night shifts would not accomplish much.

Although the Facility's current projects require performance of many analyses, it is also important that the Facility conduct research and development on analytical methodology, as funds permit. Viewing the present methodology for measuring petroleum in the marine environment, it is clear that serious gaps exist, despite recent advances. This is partly due to the substantial developmental effort which must be expended to bring methodology from the "state-of-the-art" stage to the statistically proven and accepted "standard method" stage. As part of its on-going research, the Facility is investigating applications of high pressure liquid chromatography and ultraviolet fluorescence spectrometry for the analysis of oil and its toxic constituents. These techniques should be developed into new standard methods which should then be compared with the existing ones. This applies especially to the analysis of the aromatic constituents of petroleum and their transformation products.

PUBLICATIONS

Publications listed below include those made possible by analyses performed by the Facility.

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14. POSSIBLE TRACE METAL MOBILIZATION OF SELECTED TRACE ELEMENTS FROM OIL-IMPACTED SEDIMENTS

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INTRODUCTION

Our research efforts this past year at the University of Colorado were undertaken to experimentally test whether selected trace metals can be mobilized or released from oil-impacted sediments in natural seawater. Release of trace elements from either the bottom or, less importantly, from the crude oil (the potential for trace element release from oil itself is somewhat less than the potential release from the bottom sink due to interaction with constituents of petroleum) could alter the trace element concentrations potentially available to the ocean flora and fauna. To aid us in our task, we chose four target elements to study: mercury, arsenic, chromium, and nickel. These target elements were chosen on the basis of potential toxicity and because they compose a chemically diverse group.

Mercury is of interest because it may exist in relatively unavailable inorganic forms or may be converted by microbial action to organomercury compounds that are extremely toxic. Arsenic is of interest because it exists in aqueous solution wholly in anionic form as the arsenate or arsenite, depending upon oxidizing and pH conditions. It has been shown that under conditions of low phosphate nutrient levels, aresenate inhibits active uptake of phosphate by organisms. Chromium may exist in either a cationic Cr(III) form or as hexavalent chromium(VI) oxyanions. At the conditions of pH and oxidation potential found in natural sea water, the production of chromium(VI) is favored. However, the kinetics of the chromium(III) to (VI) reaction are slow enough that it is likely that chromium(III) is being removed from water by precipitation of the hydroxide. Thus, chromium present in ocean sediments is probably a complex mixture of chromium species, all of which may be acted upon by petroleum residues in different ways. Nickel was chosen because it represents a large number of divalent transition metals that would be found in bottom sediments as hydrated metal hydroxides. Thus, in choosing these four elements for study of their interaction with marine petroleum contamination, a wide range of possible representative chemical interactions may be examined.

EXPERIMENTS

The experiments have been carried out in three phases. First, background concentrations of the target elements have been measured in seawater and sediments samples taken from Puget Sound and in Prudhoe Bay crude oil samples. The second and third phases of our experiments involved equilibrating seawater-sediment mixtures and oil-seawater-sediment mixtures. Equilibration studies involve shaking these mixtures for times from 2 days to 4 weeks.

Our initial plan of attack was to survey existing analytical techniques and concentrate on a specific method for each element which would show both high sensitivity coupled with a good dynamic range. Our choices for the analytical methodology can be summarized as follows:

1) Hg - Cold vapor Atomic Absorption Spectroscopy.

2) As - Hydride Generation Atomic Absorption Spectroscopy.

3) Ni - Graphite Furnace Atomic Absorption Spectroscopy.

4) Cr - Complexation with fluorinated β-diketone to form $Cr(tfa)_3$ and Electron Capture Gas Chromatography or Graphite Furnace Atomic Absorption Spectroscopy.

Background concentrations and concentrations of the metals in seawater for the 2-phase and 3-phase equilibrations are tabulated in Tables I-IV for all 4 elements studied. All experimental numbers have ± 10-15% uncertainties associated with them.

Unfiltered seawater was principally used for these studies. However, duplicate experiments were carried out with seawater filtered through a 0.45 μ Millipore filter. Within experimental error, the results of unfiltered vs. filtered seawater experiments appear to be the same. Both unweathered and artifically weathered crude oil samples were employed in these experiments. Crude oil samples were weathered in the laboratory by either high intensity UV irradiation or by a 3 hour ozonolysis treatment. Again, both types of samples gave similar results. The pH of the natural seawater was adjusted to pH 5.5-6.0 which represents a harsher condition than the natural environment. In subsequent experiments, the pH of the experimental seawater was adjusted to 8.0 with similar results.

Preliminary results as shown in Table I indicate that no mobilization occurred for mercury. Mercury losses from the sediment in the 2-phase experiment may be related to formation of volatile mercury complexes. The observed decrease in the concentration of mercury in the sediment for the 3-phase system is still being investigated.

Table I. Mercury Concentrations

Single Phase Experiment	
Seawater	0.5 ppb
Sediment	80.0 ppb
Two Phase Experiment (Seawater & Sediment)	
Seawater	0.4 ppb
Sediment	50.5 ppb
Three Phase Experiment (Oil & Seawater & Sediment)	
Seawater	0.1 ppb
Sediment	11.8 ppb

Table II summarizes our findings for arsenic levels in the various experimental systems. Both the 2-phase and 3-phase experiments show an increase (26.0 and 22.0 ppb, respectively) over the background level of arsenic in seawater (0.7 ppb). As the increase occurs in the shaking experiments without the oil present, we conclude that there is no mobilization occurring as a result of the contact of oil with the sediment. The measured increase in arsenic levels corresponds to only ~1% of the total concentration of arsenic adsorbed on the sediment going into solution. We suspect that the increase in arsenic is due to physical abrasion of the sediment from shaking or to slow acid leaching due to the lowered pH of these samples.

Table II. Arsenic Concentrations

Single Phase Experiment	
Oil	<0.5 ppm
Seawater	0.7 ppb
Sediment	1.5 ppm
Two Phase Experiment (Seawater & Sediment)	
Seawater	26.0 ppb
Three Phase Experiment (Oil & Seawater & Sediment)	
Seawater	22.0 ppb

Table III depicts our results for the experiments involving chromium concentrations in both the 2-phase and 3-phase experiments over background. However, since the increase occurs for the control experiment in which no oil was added, we conclude that oil-induced mobilization is not occurring. Again, the increase results from only 1% of the total chromium in the sediment going into solution.

Table III. Chromium Concentrations

	Single Phase Experiment	
Oil	----	----
Seawater	0.25 ppb 0.12 ppb	Cr(III) and Cr(VI) Cr(III) only
Sediment	3.7 ppb	Cr(III) only
	Two Phase Experiment (Seawater & Sediment)	
Seawater (8 days)	0.26 ppb	Cr(III) only
(12 days)	0.11 ppb	Cr(III) only
	Three Phase Experiment (Oil & Seawater & Sediment)	
Seawater (8 days)	0.26 ppb	Cr(III) only
(12 days)	0.18 ppb	Cr(III) only

Background levels for nickel have been determined and are presented in Table IV. The 2- and 3-phase experiments are currently in progress.

Table IV. Nickel

Single Phase Experiment	
Oil	28.0 ppm
Seawater	2.0 ppb
Sediment	11.0 ppm
Two Phase Experiments Three Phase Experiments	In Progress

Experimental problems encountered include foaming of seawater and sediment samples during mercury analysis and foaming of oil samples during arsenic analysis. To alleviate this problem, we added a few drops of 1-octanol, a known surfactant, to the samples prior to analysis. All chromium experiments must be done with glassware. Polyethylene and Teflon containers result in interferences which prohibit accurate determination of chromium concentrations. Background correction with a D_2 lamp is necessary for arsenic and nickel analyses. An automatic injection system was developed to enhance reproducibility for the arsenic method. This automatic injection system is coupled directly to the Perkin Elmer 360 Atomic Absorption Spectrophotometer. Elemental sulfur and aluminum interferences in analysis of sediment samples for chromium necessitate the use of graphite furnace AAS instead of electron capture GC detection. Figure 1 pictorially illustrates these interferences. The figure labeled sediment + benzene shows the response of the detector to sulfur. The retention time for S_8 is equivalent to that for the $Cr(tfa)_3$ complex. The sulfur can be removed by a Soxhlet extraction procedure. However, aluminum complexes with the β-diketone (Htfa) to form an $Al(tfa)_3$ complex which interferes with chromium detection. This is shown in the upper trace labeled sediment + benzene + Htfa. Thus, seawater samples were analyzed by electron capture GC and the sediment samples were analyzed using atomic absorption techniques.

CONCLUSIONS

Under the experimental conditions used, our results do not indicate any mobilization for three of our selected target metals occurring as a result of the interaction of oil upon sediments. Work is currently in progress for the remaining target element.

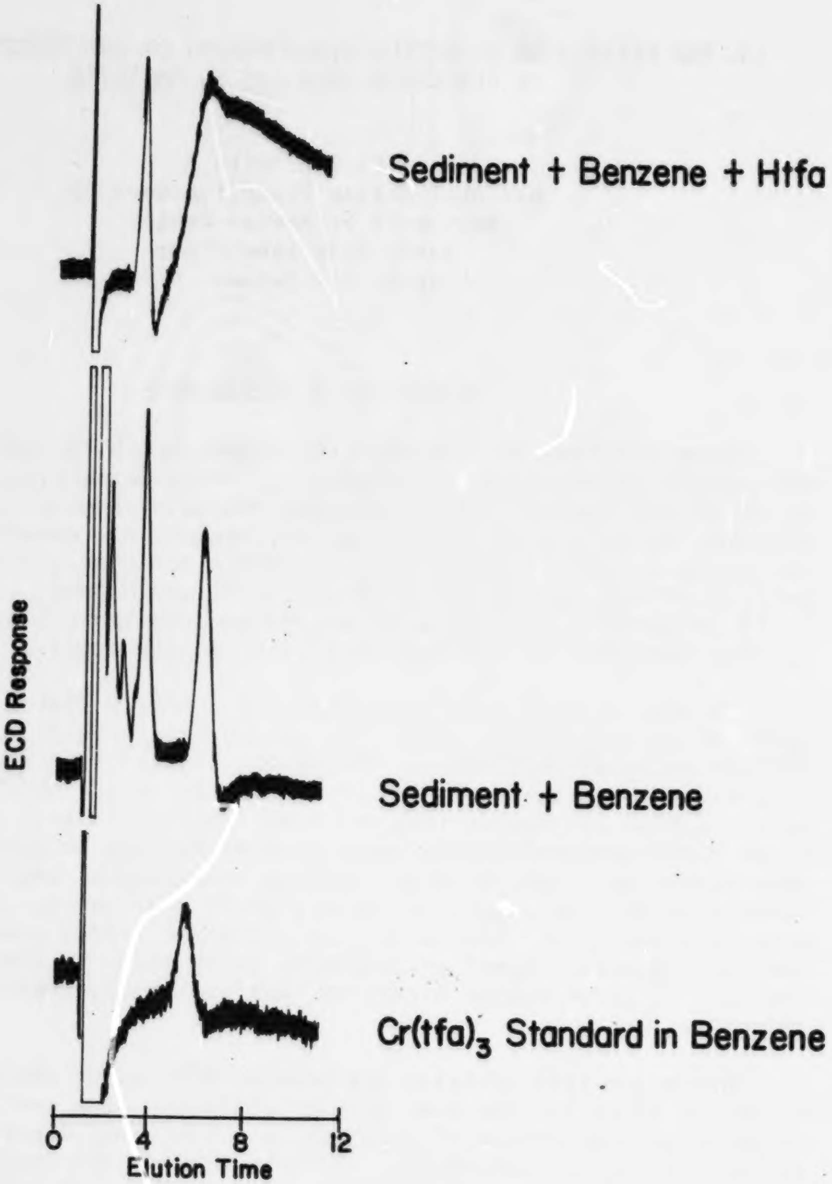

Figure 1. Interference by elemental sulfur and aluminum with gas chromatographic analysis of Cr(tfa)$_3$ complex. The figure labeled sediment + benzene shows the response of the detector to sulfur. The retention time for S_8 is equivalent to that for the Cr(tfa)$_3$ complex. The sulfur can be removed by a Soxhlet extraction procedure. However, aluminum complexes with the β-diketone (Htfa) to form an Al(tfa)$_3$ complex which interferes with chromium detection. This is shown in the upper trace labeled sediment + benzene + Htfa.

15. THE EFFECTS OF PETROLEUM HYDROCARBONS ON CHEMORECEPTION IN DUNGENESS CRAB *CANCER MAGISTER*

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OBJECTIVES AND RATIONALE

Chemoreception is a primary sense through which crustaceans gain information about their environment and, therefore, plays a central role in their life habits. Food detection and gathering, predator recognition and escape, establishment and maintenance of commensal, symbiotic and parasitic relationships, and courtship and mating are all behaviors heavily relying upon chemoreception (Grant and Mackie, 1974). Our aim ~~is~~ to investigate the effect of petroleum upon those behaviors mediated by chemoreception in the Dungeness crab, *Cancer magister*.

Because we know that an organisms's behavior flows from its perception, we can reasonably expect the crustacean's chemical sense to be influenced by the addition of chemicals. Indeed, the realization of how critical chemoreception is to success in the marine environment led Blumer (1969) to suggest that petroleum hydrocarbons in low concentrations could produce serious ecological disruption by interfering with chemoreception either through blocking reception of important chemical signals or through mimicking these signals and thereby releasing inappropriate behavior. Similarly Olla and Samet (1974) have suggested that one can logically expect petroleum to bring about sublethal effects upon behavior in those marine organisms that are highly dependent upon chemoreception.

Behaviors that mitigate any adverse effects of environmental perturbation often involve some sort of avoidance of a perturbed area. The occurrences and success of such adaptive behaviors depend upon the fulfillment of three conditions: (1) detection of the event either directly or indirectly through its environmental consequences, (2) recognition of the event as unfavorable, and (3) action that is adequate to eliminate or reduce any adverse effects. When the organism fails in its attempt to adapt, behavioral studies can still be appropriately applied to assess the effects of the perturbation. In reviewing the factors to be considered in behavioral investigations of sublethal effects, Olla (1974) indicated that the strength of the assessment and its predictive ability are directly related to the degree of understanding of the relationship between the organisms's patterns of behavior and its environmental requirements.

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Because detection of the presence of petroleum is a prerequisite to any subsequent behavior functioning to mitigate its effects, our studies with the Dungeness crab will begin with detection. The work will address four questions: (1) Can the crab detect low concentrations of petroleum? (2) When food extracts and petroleum are presented simultaneously to crabs previously unexposed to oil, is there a change in the ability of the crab to detect the food extract? (3) Is the crab's ability to detect food extracts diminished after exposure to petroleum for durations and levels consistent with realistic expectations? and (4) If petroleum exposure does diminish detection ability, how long does recovery take upon return to uncontaminated water?

METHODS

Our work on chemoreception in the Dungeness crab Cancer magister only began in October of this year. Our general approach, however, derives from our work of the previous two years upon chemoreception in the blue crab Callinectes sapidus (Pearson and Olla, 1977; Pearson and Olla, in review). Because we expect the studies with C. magister to parallel those already accomplished with C. sapidus, we shall be using the blue crab investigations as a model for our intended research with the Dungeness crab investigations. The experiments with the Dungeness crab will be based on a procedure utilizing behavioral critiera for measuring the threshold concentration at which crabs detect the food extract or other chemicals. With a standard behavioral assay, measuring the thresholds for detecting food substances for crabs subject to various kinds and degrees of petroleum exposure can be accomplished as well as measuring thresholds for the detection of petroleum and its various components. Pearson and Olla (in review) gives a description of how the behavioral assay was used to indicate detection by the blue crab of the hydrocarbon naphthalene.

MEASUREMENT OF CHEMORECEPTION THRESHOLDS

DETECTION CRITERIA

The most important step in developing the behavioral assay is the selection of appropriate behaviors as criteria indicating that the crab has detected the food extract. This step has been done successfully for the blue crab and is being done for the Dungeness crab. When a blue crab senses the food extract, there is an abrupt increase in the antennular flicking rate following within seconds by an equally abrupt onset of rhythmic maxilliped beating. These two behaviors of the blue crab are the basis of the detection criteria that can be more precisely formulated as follows: within 1½ minutes after introduction of the food extract solution the antennular flicking rate abruptly raises above 120 beats/min and rhythmic maxilliped beating commences to be continuous.

The antennular flicking rate remains continuously elevated and rhythmic maxilliped beating continues without ceasing for at least one minute after the onset of rhythmic maxilliped beating.

Using the above criteria thresholds for blue crabs detecting a clam extract were found to be about 10^{-15} g/liter and did not differ after 6 days of food deprivation (Pearson and Olla, 1977). Having detection criteria that do not require extraordinary manipulation of the crab's internal state in order to enhance their occurrence is an advantage because crabs do not have to be stressed by food deprivation, and the problem of synergism between exposure to oil and stress of food deprivation is avoided. Another advantage is that detection criteria such as that developed for the blue crab may allow us to address more directly the question of whether the inhibition of feeding following oil exposure seen in other studies derived from failure to detect sapid chemicals. The other studies presumed that the feeding inhibition is due to detection failure but could not exclude alternative explanations, e.g., that oil exposure influenced motivation to feed.

THE FOOD EXTRACT

The sapid solution presented to the crabs is a freeze-dried extract of the hard clam, Mercenaria mercenaria. The extract is preferred because single amino acids and amino acid mixtures are generally less attractive to crustaceans than extracts of natural foods are (Mackie, 1973; McLeese, 1970; Shelton and Mackie, 1971). A stock stolution of a freeze-dried clam extract (FDCE) is prepared by dissolving a weighed portion in seawater filtered through a 0.4 micron membrane. The FDCE solution is then filtered with tared Whatman No. 4 and GF/C filter paper and the concentration of the stock solution corrected for the loss of the filtrant. Fresh dilutions are prepared daily from the stock solution that can be kept refrigerated for 5 days.

The preparation of a large batch of FDCE allows the use of a sapid solution of consistent character throughout the experiment. Analyses of the chemical composition of the FDCE appear in Pearson and Olla (1977). The FDCE has worked well for C. sapidus. Clams are the major part of the diet of Dungeness crabs (Butler, 1954; Gotshall, 1977), and as anticipated the FDCE is working as well for C. magister.

THE CHEMOSENSORY TESTING APPARATUS

Dungeness crabs are presented with FDCE or other chemicals to be tested while held individually in chambers of the apparatus described in Pearson and Olla (1977). Filtered seawater of constant temperature siphons at a rate of 1.0 liter/min into a series of chambers each holding a single crab. The experimental solution is injected through a valve in the water line, and the subsequent behavior of the crab is observed from behind a blind. Dye studies are in progress in order to estimate the amount of dilution the experimental solution undergoes within a chamber.

OBSERVATION PROCEDURE

Crabs are placed into the apparatus during the afternoon and are presented with the FDCE or other experimental solution the following morning after they have habituated to the chambers. Each crab is presented with either one of a series of dilutions or a control of filtered seawater. The observer is blind to the identity of the dilutions. The order of presentation is random except that active crabs are passed over until they become still. Molting and mating crabs are not tested. The behavior of each crab upon FDCE presentation is observed and scored on the basis of criteria now being developed.

CALCULATION OF THRESHOLDS

After a number of trials at each dilution have been performed, the percentage of crabs detecting the FDCE solution can be plotted against the logarithm of the FDCE concentration (g/liter) within the chamber. The FDCE concentration at which 50% of the crabs detect the FDCE can be taken as the detection threshold and can be estimated from the graph or by regression analysis. In a similar manner thresholds for the release of feeding or detection of some other chemical can be calculated.

CHEMICAL ANALYSES AND EXPOSURE METHODS

In general the chemical analyses and petroleum exposure methods will follow those used at the Marine Research Laboratory of Battelle Pacific Northwest at Sequim, Washington. Routine measurement of oil in the delivery systems will be done with IR spectrophotometry and periodic gas chromatography. The whole oil-in-water dispersion techniques have been described by Vanderhorst et al. (1976a, 1976b).

RESULTS

The main progress in the six weeks of our work with Dungeness crab has been to confirm that changes in the orientation and flicking rate of the antennules indicate detection of food extracts by the crab. A resting Dungeness crab typically exhibits a rate of antennular flicking between 20 and 60 flicks/minute. The periodicity evident in the flicking rate of resting individuals needs further investigation. Activity increases the antennular flicking rate. When clam pieces are dropped into the aquarium or when the FDCE is presented to crabs in the chemosensory testing apparatus, the antennules change their orientation generally to an upstream direction and the flicking rate of the antennules abruptly increases to at least two times its initial rate (from 20 to 60 up to 100 to 160 flicks/minute). The doubling of the antennular flicking rate is apparently a good indication that a Dungeness crab has detected the presence of the food extract and will now be used to measure the threshold concentrations at which Dungeness crabs detect the

FDCE and seawater solutions of petroleum. Because the behavioral criteria for both *Cancer magister* and *Callinectes sapidus* are essentially the same, i.e., an increase in antennular flicking rate, the detection thresholds established for *C. magister* will give an excellent comparison of its chemosensory ability relative to *C. sapidus*.

For comparison with the Dungeness crab we can give some results of our work with the blue crab (Pearson and Olla, 1977). The blue crab has been found to have a threshold of 10^{-15} g/liter for the detection of FDCE, and this threshold did not change after six days of food deprivation. The threshold concentration at which the FDCE releases feeding behavior in the blue crab was considerably higher (10^{-2} g/liter) and did change with six days of food deprivation.

In recent work we have found that antennular flicking rate changes indicate that blue crabs can detect the hydrocarbons, napthalene and methylnaphthalene, in seawater solution. The detection threshold for naphthalene is higher than for the FDCE, and the threshold for methylnaphthalene is considered higher than that for naphthalene. Such results indicate that antennular behavior in the blue crab may be a general response to any chemical change of the crab's environment (Pearson and Olla, in review). The usefulness of behavioral assays based upon antennular motion for other crabs and a variety of chemicals, natural and artificial, seems quite promising.

PROBLEMS AND RECOMMENDATIONS

The six weeks of our study with Dungeness crabs has been too brief to encounter any significant problems. Because the behavioral criteria for Dungeness crabs are so similar to that of the blue crab, we anticipate little or no problems in measuring detection threshold for the food extract or for petroleum. Because our studies are only beginning, it is premature for us to make any recommmendations.

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16. INTERACTIVE EFFECTS OF RESIDUES OF HYDROCARBONS, CHLORINATED HYDROCARBONS, AND HEAVY METALS ON THE METABOLISM AND BIOACCUMULATION OF PETROLEUM HYDROCARBONS IN MARINE FISH

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OBJECTIVES AND RATIONALE

The principal objective of the research, since 1975, has been to provide information about the interactive effects of chlorinated hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) on the accumulation, metabolism, and toxicity of the hydrocarbons in marine fish. Since 1976, the interactive effects of heavy metals with PAHs in fish was included. There were five specific objectives of the research, as follows: (1) to determine the changes that occur in accumulation, metabolism, and distribution of chlorobiphenyls and PAHs in target organs of fish chronically exposed to those compounds separately and together in food; (2) to determine the effects of the aforementioned exposures on the activity of the xenobiotic metabolism systems of aryl hydrocarbon hydroxylases (AHH) in the microsomal fraction of fish livers; (3) to determine the interactive effects of dietary PAHs and cadmium and lead in seawater on the activity of the hepatic AHH systems; (4) to determine specific metabolic transformations of selected PAHs in vitro relative to exposures of fish to chlorobiphenyls and PAHs separately and together in food; and (5) to determine alterations in gross pathology and cell morphology of fish exposed to chlorobiphenyls and PAHs separately and together.

There are two principal aspects of xenobiotic metabolism and interactive effects of contaminants such as PAHs of petroleum that are the bases of these studies. First of all, fish possess drug metabolizing enzymes which are capable of hydroxylations of exogenous aromatic hydrocarbons (Buhler, 1966). Those enzymes, which are concentrated in liver microsomes, have been a basis for judging potential toxicities in various animals resulting from the metabolic conversions of PAHs in biological systems (Gelboin et al. 1970; Franke, 1973).

Secondly, there are data which indicate that chemicals and drugs of different classes, such as chlorohydrocarbons and barbiturates, have interactive effects with chemicals of other classes in biological systems (Burns, 1971). Simultaneous exposures, in vivo, of more than one class of chemicals can result in synergistic and antagonistic biochemical effects (Leichtenstein et al. 1973; O'Brien, 1967). In the case of marine fish which are simultaneously exposed to a variety of different classes of contaminants, it is necessary to understand (1) the fate of the contaminants in fish, (2) the possible role that one class of chemicals plays in altering the metabolism and bioactivation or detoxication of another class in fish, and (3) the ability of fish to cope with the consequences of such simultaneous exposures.

There are many hazards of chlorobiphenyls from PCBs (polychlorinated biphenyls) in marine ecosystems that are well recognized, but the role of those compounds in affecting the biochemical interactions of other contaminants in biological systems is poorly understood. It is also recognized that PCBs are very slowly metabolized or excreted from fish. Moreover, the PAHs from petroleum exposures, as well as the PCBs, are involved in the initiation of toxic processes. Thus, we hypothesize that the biochemical processes of bioactivations or detoxications of PAHs are altered by chlorobiphenyls in fish.

The metabolic products of enzyme-catalyzed transformations of PAHs are generally believed to be the toxic compounds in animals exposed to petroleum. Some of those transformations result from enzymatic hydroxylations involving arene oxide synthetase and AHH (Undefriend, 1971). Metabolic products of chlorobiphenyls in animals exposed to PCBs may also result from enzymatic hydroxylations, although considerable evidence shows that certain chlorobiphenyls, i.e., those of the highest degrees of chlorination (Matthews and Anderson, 1975), are retained as unmetabolized compounds throughout the body for long periods of time (Zitko, 1971; Gruger et al. 1975). However, there is evidence that PCBs in vivo induced the AHH activity in hepatic microsomes in vitro of fish exposed to PCBs in food (Gruger et al. 1977a). Therefore, metabolic products of both PAHs and PCBs may be affected by the biochemical interactions of chlorobiphenyls in fish.

In regard to heavy metals, such as cadmium and lead, they have been shown to cause changes in activities of several liver enzymes in fish (Jackim et al. 1970). If fish are exposed to heavy metals and aromatic hydrocarbons at the same time, then perhaps the metabolism of the hydrocarbons may be altered. Hence, it is important to determine the possible interactive effects of heavy metals that may occur in fish which are exposed to PAHs, in order to predict additional impacts on the bioactivation or detoxication of PAHs in fish.

Early stages of biological change, manifested by alteration in cell morphology, may precede and pinpoint later toxic events in exposed organisms. In addition, morphology provides clues about xenobiotic processes in cells that may be correlated with specific biochemical tests. For example, proliferation of the endoplasmic reticulum in isolated organs and cells has been associated with induced synthesis of enzymes of xenobiotic metabolism (Ernster and Orrenius, 1973). Also, microscopic evidence for disruption of cells, tissue membrane irregularities, and mucosal sloughing in intestine, among other morphological observations, are strongly indicative of possible harmful effects of a contaminant in animals (Gardner, 1975; Hawkes 1977a, b; Couch 1977).

METHODS

CHRONIC EXPOSURES OF SALMON TO CHLOROBIPHENYLS AND HYDROCARBONS

Salmon were maintained in pens located in seawater and were fed specifically prepared food consisting of Oregon moist pellets (OMP) which contained mixtures of model compounds of chlorobiphenyls and petroleum hydrocarbons. The mixture of chlorobiphenyls consisted of biphenyl, 2-chlorobiphenyl, 2,2'- and 2,4'-dichlorobiphenyl, 2,5,2'-trichlorobiphenyl, and 2,5,2',5'-tetrachlorobiphenyl. The mixture of hydrocarbons consisted of nearly equal amounts of 2,3-benzothiophen, n-pentadecane, 2,6-dimethylnaphthalene, 2,3,6-trimethylnaphthalene, fluorene, phenanthrene, 1-phenyldodecane, and heptadecy/cyclohexane. The OMP included the chlorobiphenyls and hydrocarbons at 1 and 5 ppm in separate preparations and together in the ratio of 1:1 (wt/wt) at 2 and 10 ppm. The food-path exposures were performed in seawater with coho salmon (Oncorhynchus kisutch) in duplicate series. The exposures lasted for 4 weeks, followed by a 4-week depuration period during which all fish received control food.

The coho salmon which ranged in initial weights from 184 ± 41 to 204 ± 41 g in 14 groups of 150 fish per group, were exposed from February to April 1976. Similarly, approximately 600 chinook salmon (O. tshawytscha), which ranged in initial weights from 72 ± 14 to 76 ± 16 g in separate groups, were exposed (without a duplicate series) in July and August 1976.

At various intervals during the exposures and the depuration period, fish were taken for analyses of kidney, liver, and body tissues remaining after removal of the visceral organs, head, and tail. Concentrations of parent hydrocarbons and chlorobiphenyls were determined, by gas chromatography, in the tissue samples of the coho salmon which received the highest concentration regimes of xenocompounds. The analyses were performed by the NOAA National Analytical Facility (NAF), with staff assistance (notably John Finley) from the Environmental Conservation Division, Northwest and Alaska Fisheries Center. The sample handling and extraction procedures involved in the analyses were those reported by Macleod et al. (1976). The routine limit of detection for each chlorobiphenyl and hydrocarbon was 20 ng/g dry wt of tissue.

Analyses of specific activities of hepatic microsomal AHH were performed by Dr. Marleen Wekell and Neil Stewart, using procedures adapted to those reported by DePierre et al. (1975) and optimized for the two species of salmon. Specific activities of AHH were based on carbon-14 labeled benzo[a]pyrene as a substrate, and on protein concentrations of the microsomal fractions which were standarized to bovine serum albumin (as in a Lowry method). Freshwater rainbow trout (Salmo gairdneri) were used to provide hepatic microsomal fractions, which served as reference material for monitoring the consistency of the analytical methods for determining AHH activity.

Statistical comparisons of AHH activities were made with the Student's t-test. P values less than 0.10 were considered to represent significant differences between mean values.

EFFECTS OF PAHs AND HEAVY METALS ON AHH ACTIVITIES

Coho salmon and starry flounder (Platichthys stellatus) were preconditioned in seawater containing 200 ppb of cadmium or lead for 10-14 days, in order to allow time for the heavy metals to perhaps perturb the hepatic AHH sytems in the exposed fish. During the subsequent final two weeks, while the metal exposures continued, the fish were fed OMP which contained 334 ppm of a model mixture of PAHs. The mixture consisted of nearly equal proportions of phenanthrene, 2-methylnaphthalene, and 2,6-dimethylnaphthalene. After the final two weeks, the fish were taken for analyses of AHH activities in hepatic microsomal fractions. The controls were fish that received an OMP diet without the PAHs. The AHH activities were determined by Dr. Jerome Schnell on six groups of seven to ten fish per group for each species. Activities of the AHH, as naphthalene hydroxylases, were determined by the method of Nilsson et al. (1976).

An assessment of the effects of the heavy metals on the microsomal AHH activities in an in vitro system was made. Cadmium and lead concentrations were separately varied from 0.05 to 500 ppm in enzymic reactions. The sources of microsomes were pooled samples of liver fractions from the coho salmon fed chlorobiphenyls and hydrocarbons in the chronic exposure studies.

METABOLIC TRANSFORMATIONS OF PAHs IN VITRO

The naphthalene family of PAHs, being important components of seawater-soluble fractions of petroleum, are presently under study by Dr. Jerome Schnell and Lucinda Grant. Determinations are being made of metabolic transformations of naphthalenes that may be affected by biochemical interactions of PCBs and PAHs in fish. Radiochemically labeled substrates for hepatic microsomal enzymatic hydroxylations are being used in vitro. A method employed for analysis of AHH activity (Nilsson et al. 1976) was scaled up to use greater amounts of naphthalenes, and reaction times were extended so as to assure significant quantities of metabolites for extractions and quantitations. At the same time, a preliminary determination of specific activities of AHH, based on benzo[a]pyrene, naphthalene, and 2,6-dimethylnaphthalene, was made so as to predict relative differences that would occur in the formation of metabolites from different substrates.

CELL MORPHOLOGY OF FISH EXPOSED TO CHLOROBIPHENYLS AND HYDROCARBONS

Coho salmon fed diets (described above) containing the hydrocarbons and chlorobiphenyls, alone and together, were sampled 16 and 28 days after initiation of feeding, and again after 21, 28, and 42 days of depuration. Samples of skin, gills, and intestine were processed for scanning electron microscopy; and skin, gill, liver, and kidney for transmission electron microscopy (Hawkes, 1974). In the early sampling, five blocks of each tissue from two fish were prepared and embedded in plastic. In subsequent sampling, tissues from three fish from each exposure type and concentration were used.

The experiment was repeated using chinook salmon, and samples of the same tissues were prepared 3, 18, and 28 days after initiation of feeding and after 22 days of depuration. One micron-thick sections for light microscopy were cut with glass knives and stained with Mallory's Azure II-methylene blue (Richardson et al. 1960).

PATHOLOGY OF FISH EXPOSED TO CHLOROBIPHENYLS AND HYDROCARBONS

The gross physical condition of the fish during the chronic feeding exposure study were monitored for signs of pathology by observing the possible presence of kidney lesions, erosions of fins, changes in hemocrits, and mortalities. In addition, enlargement of spleens and the presence of orange pigmentations of livers were monitored as indications of possible abnormalities of the experimental animals.

RESULTS.

DISTRIBUTION OF CHLOROBIPHENYLS AND HYDROCARBONS IN COHO SALMON

Chemical analyses of body tissues (principally muscle), kidney, and liver from the coho salmon revealed chlorobiphenyls in each tissue in 1 to 4 weeks of exposures and up to 4 weeks of depuration. All of the chlorobiphenyls were found in the three tissue sites from fish fed the chlorobiphenyls both separately and together with the hydrocarbons. The concentrations tended to be greater in muscle than in kidney and liver as time progressed. Biphenyl and 2-chlorobiphenyl appeared to be absent in the tissues at earlier times, i.e., from 3 to 4 weeks, while the biphenyls of greater chlorination tended to remain to the end of 8 weeks of sampling. There was no evidence for the influence of dietary aromatic hydrocarbons on the tendency of fish to accumulate the chlorobiphenyls.

Tissue samples of control fish were found to contain n-pentadecane and 1-phenyldodecane; therefore, those chemical were disregarded due to their interference in the anlyses. However, no evidence was found for the other hydrocarbons in the muscle, kidney, and liver from the salmon fed the hydrocarbons separately or together with the chlorobiphenyls.

There are several conclusions about the study of the chlorobiphenyls and hydrocarbons in the exposed fish, as follows: (1) The fish did in fact ingest the hydrocarbons because the analysis of fish which received the 1:1 mixture of hydrocarbons and chlorobiphenyls showed that the chlorobiphenyls were in the tissues; i.e., the chlorobiphenyls served as marker compounds. (2) The PAHs in the diet are not found at $\geq$ 20 ppb, then they must be in the form of metabolites and/or membrane-bound conjugates (which were not measured). (4) The hydrocarbons were either metabolized or not absorbed in the digestive tract and transferred to the circulatory system of the fish like the chlorobiphenyls.

EFFECTS OF CHLOROBIPHENYLS AND HYDROCARBONS ON AHH ACTIVITIES

The AHH activity was induced within two weeks in the coho salmon by hydrocarbons administered at 5 ppm, with and without chlorobiphenyls in diets. The activities of the AHH were as great as 1.9 times the activities for the corresponding control coho salmon ($P<0.01$), after one week exposures to the 10 ppm mixture of hydrocarbons and chlorobiphenyls. With 1 ppm hydrocarbon exposures, there were no significant differences compared to controls. The relative AHH activities for the first two weeks of exposures are illustrated in Figs. 1 and 2.

With chinook salmon that were fed hydrocarbons alone, the hepatic AHH activity was significantly ($P<0.05$) depressed. The depression of AHH activity began after 2 days of exposure and continued for 21 days, with the exception of the third day when analyses showed no effect. When the salmon were exposed to hydrocarbons together with chlorobiphenyls, depressed AHH activity ($P<0.05$) was found at days 1, 3, 7, and 21; at days 2, 4, and 14 there was no effect on AHH activity. The latter results are analogous to the variation in AHH activity shown for *Pleuronectes platessa* exposed to dieldrin in seawater (Vink, 1975).

Chlorobiphenyls alone did not cause significantly increased hepatic AHH activity in the coho salmon (Figs. 1 and 2). That finding is contrary to results with yearling coho salmon in fresh water (Gruger et al. 1977a). For chinook salmon, chlorobiphenyls alone significantly ($P<0.05$) depressed AHH activities at all times up to 21 days, except for samples taken on days 2 and 14 when the activities were statistically indistinguishable from controls.

An outstanding feature of the results of AHH analyses was the wide variation in individual values for the enzyme activity for cohorts of each experimental group. For example, the standard deviations varied from ±23% to ±90% of the mean values for the first two weeks of the coho salmon exposures. Similar wide variations were found for the chinook salmon hepatic AHH activities *in vitro*. The wide variations are illustrated in Figs. 1 and 2.

Evidence was found for a shift in the optimum pH of hepatic AHH activity *in vitro* for chinook salmon used in the experiments. The control fish, the chlorobiphenyl-fed fish, and the fish fed the mixture of chlorobiphenyls and hydrocarbons had an optimum pH of 7.8 for optimum AHH activity *in vitro*. The fish fed hydrocarbons alone, however, had an optimum pH of 7.8 up to about 3 days of exposure, after which time a

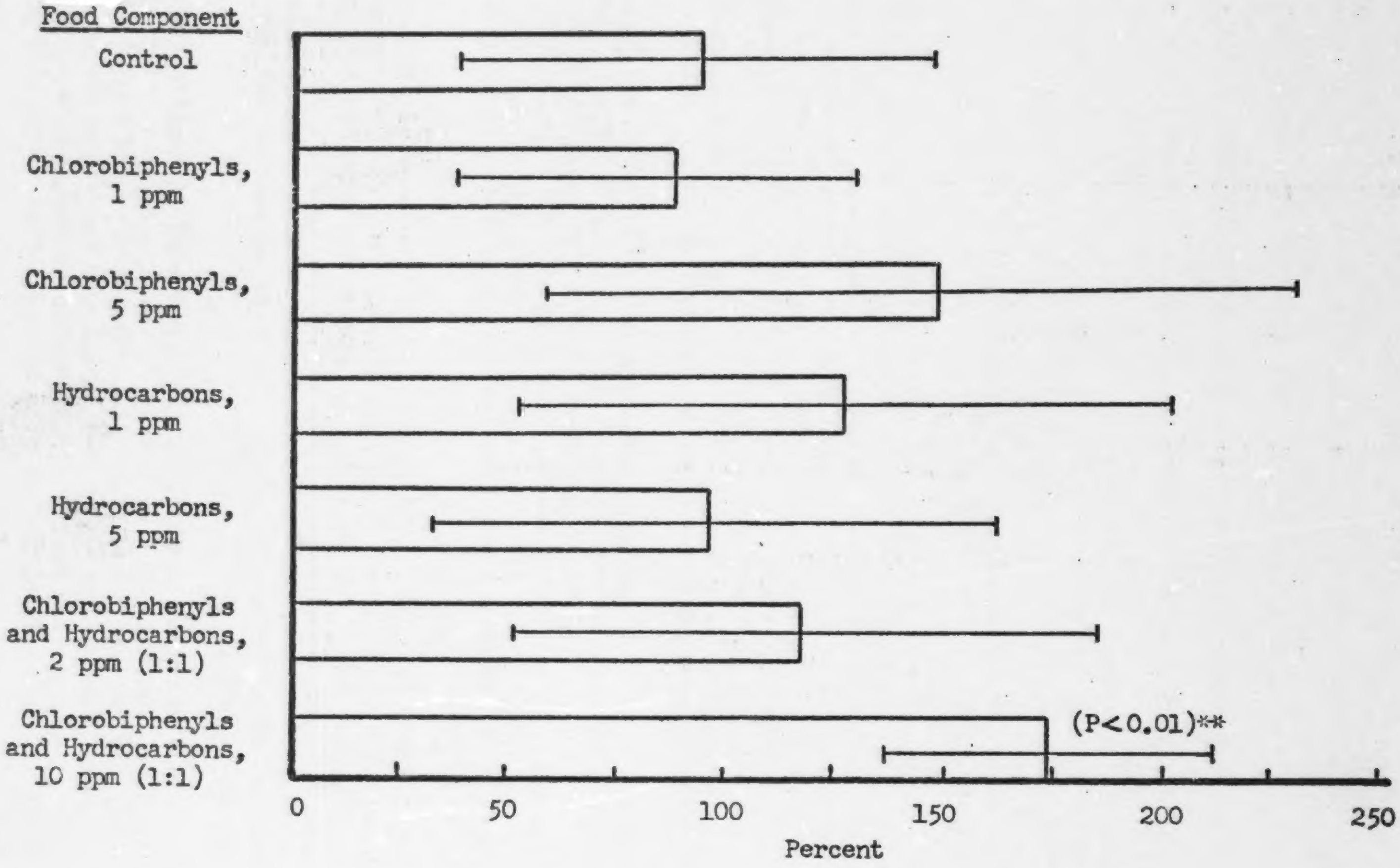

Figure 1. Relative hepatic aryl hydrocarbon hydroxylase activities, after one week of administration of chlorobiphenyls and petroleum hydrocarbons in food to coho salmon (Gruger et al. 1977b). Lines (├─┤) represent standard deviations. **Compared to control.

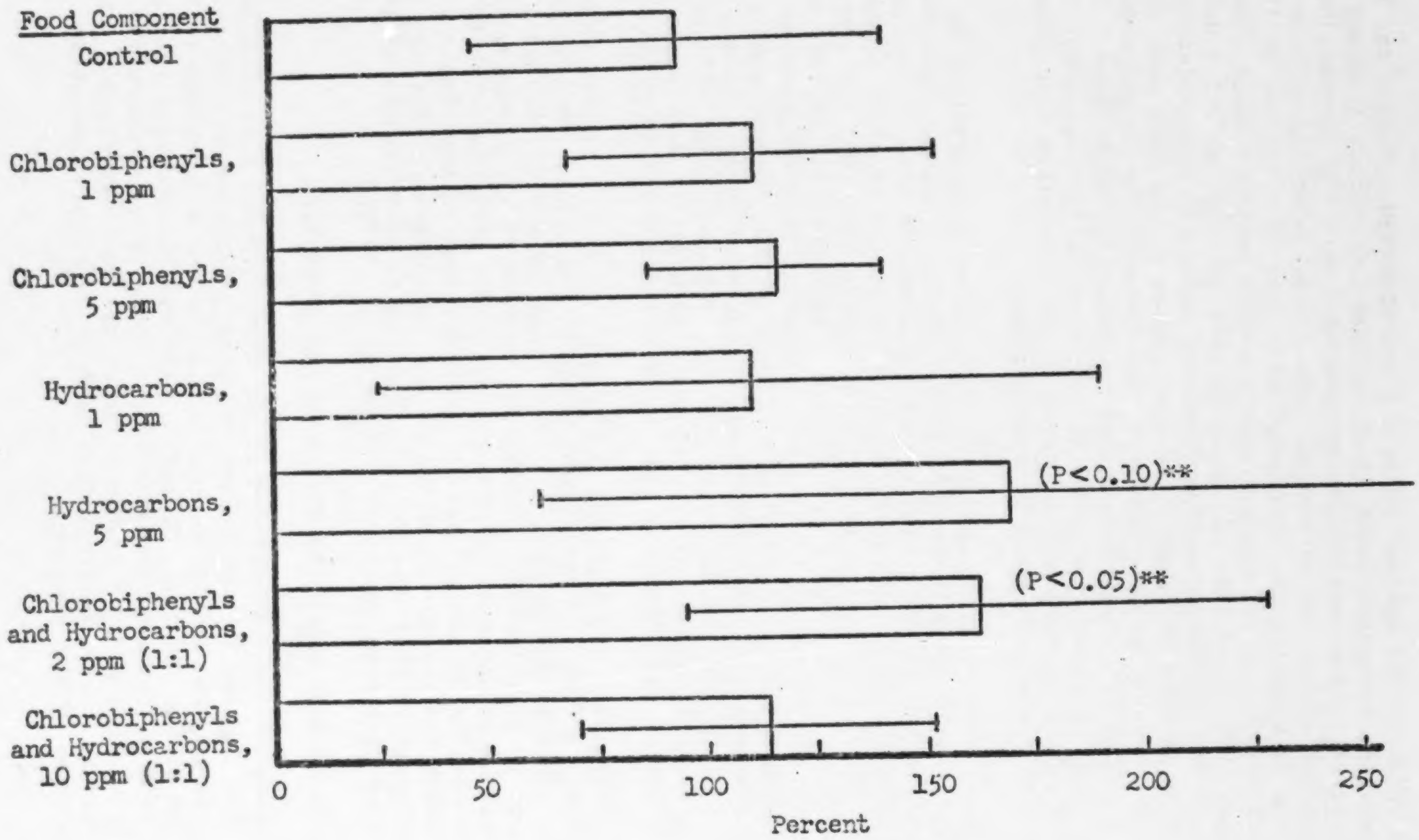

Figure 2. Relative hepatic aryl hydrocarbon hydroxylase activities, after two weeks of administration of chlorobiphenyls and petroleum hydrocarbons in food to coho salmon (Gruger et al. 1977b). Lines (⊢⊣) represent standard deviations. **Compared to control.

downward shift in the optimum pH to 7.5 was observed. Hanson and Fouts (1971) reported an upward shift in pH optimum for aniline hydroxylase activity *in vitro* for rats following treatment with PAH (benzo[a]pyrene); it was concluded that the microsomal type I substrate-binding site was involved in determining the optimum pH of a type II substrate of the microsomal mixed function oxidase system (which includes AHH). The preliminary indication from our study is that the PAHs affect a different part of the xenobiotic system involving enzymatic hydroxylations than do PCBs in chinook salmon. In this instance, the same part of the enzymic system seems to be associated with the exposure to chlorobiphenyls together with hydrocarbons and to chlorobiphenyls alone. Conceivably, the two classes of chemicals together in fish may alter the metabolism of the hydrocarbons compared to the metabolism that occurs when the fish are exposed only to hydrocarbons.

Evidence was found for an apparent synergistic interaction *in vivo* of chlorobiphenyls and hydrocarbons that correlated with a significant ($P<0.05$) induction of AHH activity *in vitro* after 14 days of exposure in the coho salmon. At a concentration of 1 ppm of the separate classes of compounds, there was no alteration of the enzyme activity, but a significant induction occurred when the compounds were together at that concentration, i.e., 2 ppm of 1:1 mixture. However, only the hydrocarbons at 5 ppm induced the activity of AHH. These results suggest that a substrate concentration near 1 ppm must be exceeded *in vivo* before activation of the AHH systems by PAHs can be detected. The effective concentration that perhaps must be exceeded in order to detect the activation may be different for each xenobiotic chemical *in vivo*.

The conclusions are that the two species of salmon exhibit different responses to PCBs and hydrocarbons, which are mainly PAHs, in terms of hepatic AHH activity *in vitro*; that chlorobiphenyls may interact with hydrocarbons to accentuate the xenobiotic enzymes in coho salmon; and that either chlorobiphenyls of PAHs may interfere with the activity of xenobiotic enzymes in chinook salmon. The interactive xenobiotic effects of these two classes of chemicals may be influential factors in marine environments that contain these chemicals. The results of the above studies have been published (Gruger et al. 1977b).

INTERACTIONS OF PAHs AND HEAVY METALS ON AHH ACTIVITIES

The study on the interactions of the dietary model PAHs with cadmium and lead exposed to coho salmon and starry flounder in seawater showed no significant effect on hepatic naphthalene hydroxylase activity *in vitro* for either species. Therefore, the results indicate that the metabolic hydroxylation of aromatic hydrocarbons is not necessarily mediated by cadmium or lead exposures under the test conditions. This study is carried out in collaboration with Dr. William Reichert and studies under an OCSEAP project.

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Other experiments were carried out with cadmium and tritiated naphthalene in incubation mixtures with hepatic microsomal fractions from coho salmon and rainbow trout. The results showed that 5 ppm ionic cadmium caused inhibition of naphthalene hydroxylation. Lead was similarly found to inhibit the same AHH system *in vitro*, but at a concentration of at least twice that of cadmium.

The absence of correlations between the results of experiments *in vivo* and *in vitro* indicates that the fish have the means to sequester the heavy metals or otherwise render them innocuous to AHH and not allow heavy metal ions to reach the sites of AHH activity within hepatocytes.

METABOLISM OF PAHs IN VITRO

Protocols for metabolic rations *in vitro* were established for isolations of metabolites of naphthalene and methyl-substituted naphthalene. A pure reference compound, 1,2-dihydronaphthalene-1,2-diol, was synthesized and used in analyses. Attempts are being made to correlate molar ratios of monohydroxy naphthalenes to the corresponding dihydrodiols from reactions of microsomal enzymes of salmon, which were exposed to chlorobiphenyls and hydrocarbons. Changes in the ratio of two metabolites formed under steady-state kinetic conditions may be indicative of the multiplicity of microsomal enzymes (Trager, 1977).

Thus far, there is evidence from our studies that the chlorobiphenyls alone or together with the hydrocarbons *in vivo* cause perturbations of microsomal enzymes which catalyze the hydroxylations of aromatic hydrocarbons *in vitro*. There appears to be marked differences in AHH activities *in vitro* for a given microsomal fraction of fish liver, depending on the type of enzyme substrate, such as benzo[a]pyrene, naphthalene, and 2,6-dimethylnaphthalene (1,6-DMN). This work is continuing. In preliminary experiments, however, there appears to be species differences, inasmuch as coho salmon microsomes from PCB- and PAH-exposed fish were found to cause greater proportions of 2,6-dimethylnaphthol (tentatively identified) *in vitro* in relation to a more polar metabolite (probably a dihydrodiol) than did the corresponding chinook salmon microsomes. The results of the study are expected to show whether chlorobiphenyls promote a preferential formation of one type of naphthalenic metabolite, rather than another type, when fish are exposed to the chlorobiphenyls simultaneously with PAHs. By knowing the identity of the principal metabolites formed under different conditions, biologists can begin to direct their studies at the fate of those major components derived from contaminants.

PATHOLOGICAL OBSERVATIONS OF SALMON IN EXPOSURE STUDIES

Some mortalities were observed throughout the food-pathway exposure experiments, but these could not be attributed to the composition of the diets. At the beginning of the study, all salmon appeared healthy. However, all test coho salmon exhibited random and progressive signs of fin lesions (fin rot disease), kidney lesions, enlarged spleens, and organ pigmentations of livers during the course of the study. Moreover, the control coho salmon appeared generally healthier than the salmon in the test groups. At the eighth week of sampling for AHH analyses, all fish exhibited the abnormal health signs.

Hematocrits of coho salmon, measured during the fourth and fifth weeks, ranged from 4 to 52%, with mean ± S.D. values of 34.3 ± 12.2% and 31.8 ± 14.0%, respectively. On the whole, average values of hematocrits appear to be poor indicators of the presence of pathology; however, individual hematocrits of 20% and less may be indicative of a forthcoming disease condition in the fish.

A recalculation of hepatic AHH activities for coho salmon based on visible signs of good health versus pathological symptoms did not change the results and interpretations.

All chinook salmon were in excellent condition at the start and no signs of pathology were evident until after one week of feeding, when one sample fish showed signs of fin erosion. It was not until four weeks of exposures to the test compounds that kidney lesions were observed. In general, the chinook salmon were healthier than the test fish.

Measurements of hematocrits were also made for the chinook salmon. These measurements were for all chinook salmon taken for the analyses of AHH activity, because of our concern for the condition of the fish after our experience with the coho salmon. Hematocrits ranged randomly from 25 ± 11% to 40 ± 10% among groups with no apparent relationship to pathology.

Many factors affect hematocrits or blood cell counts in fish, such as water temperature, state of maturity, spawning cycle, metabolic activity and sampling techniques. If we assume that values of 32 to 52% found for juvenile hatchery coho salmon (10-15 g size) are reasonable values for mature salmon in seawater (Wedemeyer and Chatterton 1971), then perhaps the salmon with hematocrits of about 30% can be regarded as bordering on a health problem.

MICROSCOPIC EXAMINATIONS

Skin and gill tissues from coho salmon exposed for three and four weeks to each dietary concentration of xenocompounds were examined with the scanning electron microscope (SEM). No differences were noted in the gills. In the skin, there were areas of sloughed and necrotic cells in both control and experimental salmon; however, differences were evident in surface skin cells of fish exposed to the 10 ppm mixture of hydrocarbons and chlorobiphenyls. After three weeks of exposure, the surface membrane, which forms ridges in normal salmon, was irregular, and the areas of sloughed cells appeared larger. Disruption of the surface cells was even more pronounced in the experimental samples taken after four week of exposure to 10 ppm mixed hydrocarbons and chlorobiphenyls; it was questionable whether the hydrocarbons or the chlorobiphenyls alone were deleterious.

Hepatocytes from the 5 ppm chlorobiphenyls-treated chinook salmon were highly vesiculated and foam-like in structure compared to control fish. Similar "foam-like" changes were reported in liver cells of rats which received 500 ppm PCB-supplemented diet for seven months (Itokawa et al. 1975). Additional liver changes in rats, rabbits, and guinea pigs include lesions, fatty infiltration and necrosis (Fishbein, 1974). In both mice and monkeys fed chlorinated biphenyls, light microscopy revealed slight liver changes but transmission electron microscopy (TEM) showed "marked alteration" in hepatocytes--the smooth endoplasmic reticulum being increased (Fishbein, 1974). In addition to vesiculated cells, the chinook treated with 5 ppm chlorobiphenyls had inclusions of intracellular whorled membranes. Both of these cellular anomalies resemble the cell types described by Bannasch (1976) from rat neoplastic nodules. The only abnormality that was evident in the liver samples from two other chinook treated with 5 ppm chlorobiphenyls was a slight increase in fat vacuolization compared with the controls. Light microscopy of chinook liver cells from exposure regimes other than 5 ppm chlorobiphenyls revealed no differences from the control fish.

There were sloughed and necrotic cells in the anterior intestines of chinook fed 5 ppm chlorobiphenyls and 10 ppm mixture of hydrocarbons and chlorobiphenyls. Necrotic areas were scattered throughout the sections and, in many instances, were adjacent to areas with normal morphology. More extensive areas of damage were evident when hydrocarbons and chlorobiphenyls were administered together than when xenobiotic alone was administered.

In the affected intestinal cells there were several degrees of damage which ranged from vesiculation inside the surface cells to sloughing of entire cells. When PAH alone was administered, there was some sloughing at the surface of the intestinal mucosa which was clearly not present in the control fish but a less pronounced effect than in the other experimental exposure regimes.

Two of the 5 ppm chlorobiphenyl-treated chinook had pronounced vacuolization in the intestinal mucosal cells and TEM examination confirmed that the vacuoles contained lipid. In addition, some mucous glands were notably smaller, the mucin was frequently not sequestered in the typical membrane-bound vesicles of control fish. Other mucosal cells that normally do not exhibit secretion products had large vacuoles full of amorphous material which has not yet been characterized histochemically, but morphologically, and appears to be mucin.

With both PAH and chlorobiphenyls together, the mucous glands are much less conspicuous and fewer cells have disorganized mucin-filled vacuoles but this is still a common abnormal response. The mucosal cells are generally smaller than in control fish and cuboidal rather than the usual elongated cylinder. There was pronounced increase in endoplasmic reticulum and mitochondria in the cuboidal cells. Desquamation of the mucosa was more pronounced in the combined PAH and chlorobiphenyl exposure.

SEM examination of portions of the intestinal surface was equivocal: so much debris was on the surface of the experimental specimens that cell surface structure was obscured. Damage resulting in sloughing of the intestinal epithelium certainly could account for this observation.

PROBLEMS ENCOUNTERED

In the food-path exposure experiments, the coho salmon turned out to be of poor quality. Morphology indicated that the tissues of the control animals were in bad shape. General appearances of kidney lesions, pigmented livers, and fin erosion among the coho salmon after 3 to 4 weeks was reason to negate interpretations of effects on AHH activities after that time. These problems were also the reason to repeat the exposure study with the chinook salmon, which fortunately or unfortunately were the only salmon available for our use. The chinook salmon were in excellent condition when they were used initially; however, they too showed signs of health problems, but much later in time than did the coho salmon. Reasons for the problems with chinook salmon can only be speculative.

The unavailable pure standards of metabolites of 2,6-DMN and other naphthalenic compounds is a problem for absolute identifications. We plan to do more syntheses of reference chemicals, such as dihydrodiol of 2,6-DMN, to serve these purposes.

RELEVANCE OF PROBLEMS OF ENVIRONMENTAL CONTAMINATION

There are three aspects of the research that are especially relevant to problems of environmental contamination. These relate to (1) the use of AHH activity as an index for monitoring petroleum pollution (Payne 1976), (2) the selection of hydrocarbons for substrates in the determinations of AHH activities and the environmental implication of the findings, and (3) the apparent synergism of chlorobiphenyls and PAHs in altering AHH and cell morphology in salmon. Each of these are discussed in order.

AHH ACTIVITY AS A MONITORING INDEX

There were wide variations in the values of AHH activities for cohorts of experimental and control fish. The wide variations in the enzyme activities may require such a large number of samples that the application of the method for monitoring may have little practical value and lack feasibility. Also, as our results showed, when exposure periods extend beyond two weeks, the values of AHH activities for exposed coho salmon tend to become quantitatively similar to the controls; this tendency could preclude the application of AHH activities to monitoring of oil pollution. However, in cases where inductions of AHH activities occur in less than two weeks, then the exposure period may not be a negative factor.

In addition, species differences may limit the use of AHH activity as a monitoring index. As we found, chinook salmon had depressed AHH activities under conditions when coho salmon had elevated or induced AHH activities.

The modes of exposure of fish to petroleum must indeed be considered a factor in judging whether AHH activities are suitable monitoring indices. It must be borne in mind that the present work involved food-path exposures, which relate to food-web transfers of contaminants and to adsorptions to planktonic particles which may be ingested by fish. Petroleum dispersions in seawater may be the more obvious mode of exposures, for which AHH activities were originally proposed for monitoring oil pollution (Payne, 1976), but those, too, cannot be free of the problems just mentioned.

HYDROCARBON SUBSTRATES FOR AHH

Our finding that different values of AHH activities result from different aromatic hydrocarbon substrates in the enzymatic analyses means that the individual PAHs of petroleum may be metabolized in fish at different rates. That situation can partly account for wide variations found in the profiles of aromatic hydrocarbons retained in fish exposed to equal concentrations of hydrocarbons. This has important implications, because often hydrocarbon uptake experiments are performed with the test hydrocarbons at equal concentrations, in order to determine differences in retentions of hydrocarbons. In addition, the different AHH activities may, in turn, account for differences in the rates of elimination of metabolites of the hydrocarbons in fish.

The differences in AHH activities associated with the various PAH substrates could be the basis for selection of a substrate that provides for higher AHH activities (as with 2,6-dimethylnaphthalene) than substrates used in the past such as benzo[a]pyrene, aniline, and acetanilide (Payne, 1976; Buhler and Rasmusson, 1968). In fact, the benzo[a]pyrene hydroxylase system has several disadvantages including poor response to drug-metabolizing enzyme inducers (Fouts, 1973). When AHH activity is employed for determining the impact of particular PAHs on biological systems, logical substrates for determining AHH perturbations might well include the class of PAHs under study. For example, 2,6-dimethylnaphthalene is the most sensitive substrate thus far identified for use in studies of the effects of the naphthalenic class of hydrocarbons in fish.

APPARENT SYNERGISM OF CHLOROBIPHENYLS AND PAHs

Some important results of our studies relate to the interactive effects of chlorobiphenyls and PAHs in salmon. Independent evidence was found for apparent synergism in induced enzyme activities from fish liver microsomes and the changes in morphology in the fish. The present work provides the first description of morphological changes which show a synergism in the effects of two contaminants on a marine fish. The enhancement of the deleterious morphological effects of petroleum hydrocarbons and chlorobiphenyls at low concentrations has potentially serious ramifications in considering the impact of water- or food-borne contaminants on fish. The findings suggest that multiple contaminants consisting of different classes of chemicals in a food source may perturb biological systems to a different degree than what might be found for a single class of chemicals or contaminant. The significant multiple effects must logically be taken into account when examining effects of different contaminants in the environment and in attempting to make balanced judgments of contaminant effects.

CONCLUSIONS

The present research has uncovered some important factors that deal with interactive biochemical and morphological effects of contaminants in organisms in the marine environment. We must conclude that species differences, wide variations among individual fish to respond biochemically to contaminants, and the potential for simultaneous interactions of chemicals from more than one class are factors which must be seriously considered in attempting to resolve environmental problems. The research keenly demonstrates the advantage of a multidisciplinary approach to defining effects of contaminants.

The research specifically leads to the conclusion that chlorobiphenyls may either enhance or retard the normal biotransformations of petroleum aromatic hydrocarbons in exposed fish, in terms of responses of the hepatic microsomal AHH systems in chinook and coho salmon. Moreover, morphological changes occur in salmon as a result of food-path exposures of chlorobiphenyls and hydrocarbons; the changes appear less severe when the chemical classes are administered separately to the fish. Importantly, synergistic action by the two types of chemicals were apparent in morphological changes and increases in liver AHH activities for exposed salmon. Our finding varied AHH activities relative to different substrates has important implications about the metabolism of petroleum hydrocarbons.

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RECOMMENDATION FOR FUTURE RESEARCH

Future research under NOAA's Energy Resources Project on Fate and Effects of Petroleum Hydrocarbons should continue studies on biochemical effects when multiple contaminants are present. The work might reasonably include testing for environmental effects with petroleum compounds and organic chemicals which are recognized in the Toxic Substances Control Act. A Federal panel of interagency scientists, in January 1977, recommended closer examinations of certain compounds and specified a list that included four individual chemicals and six classes of chemicals (cf. Chemical and Engineering News, October 24, 1977). That list includes the class of cresols, which are hydroxylated toluenes. Hydroxylated aromatic hydrocarbons are one of several classes of compounds expected to occur in the environment because of oxidative weathering processes that can act on petroleum in the environment. The metabolism of aromatic hydrocarbons includes the formation of hydroxylated hydrocarbons, which may be represented by the cresols. The simultaneous presence of hydroxylated aromatic hydrocarbons or cresols and petroleum hydrocarbons may produce different environmental effects in marine organisms than the hydrocarbons by themselves. Therefore, our recommendation is to determine the interactive effects of specific petroleum hydrocarbons and cresols on the metabolism of the hydrocarbons in marine organisms and to interrelate pathology and morphology of the organisms to exposure regimes of those chemicals and to the appropriate biochemical processes, where possible. In addition, future research should perhaps include the other recommended classes of chemicals which come under the Toxic Substances Control Act.

Cellular responses appear to be very sensitive indicators of the response of fish to toxic materials and some effects appear to be specific to the contaminant and some appear to be common to any toxic material. Since we now have an understanding of the cellular effects of PAHs and chlorobiphenyls alone on several fish tissues, as well as evidence for a synergistic effect in the two compounds affecting damage to the intestine, other compounds which might be found in the same environmental areas as PAHs chlorobiphenyls should be tested for individual and additive effects. Future research should include further testing of morphological criteria for assessment of tissue damage followed by identification and separation of specific and general effects of different classes of contaminants.

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17. DESIGN OF FIELD EXPERIMENTS TO DETERMINE THE ECOLOGICAL EFFECTS OF PETROLEUM IN INTERTIDAL ECOSYSTEMS

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OBJECTIVES AND RATIONALE

Is it possible to design a field experiment that will yield ecologically and statistically significant information about how oil affects intertidal ecosystems? What classes of experimental design and technical approach are most likely to generate optimal information on these effects? Such questions arise in the process of gathering information to enable prediction and assessment of impacts of oil and gas developments on marine environments. Information is needed to support decisions regarding the exploration and development of offshore petroleum resources; and regulations must be established regarding leasing, operating and monitoring which permit resource developments with maximum environmental protection. In order to generate this information, the National Oceanic and Atmospheric Administration (NOAA), with support from the Environmental Protection Agency (EPA), is conducting studies on the Fate and Effects of Petroleum Hydrocarbons and Selected Toxic Metals in Marine Organisms and Ecosystems. This paper highlights and summarizes the results of one small part of this program aimed at answering the questions raised above. The work reported herein is described in detail in Moore and McLaughlin (1977).

A primary goal of the field experiments in question is to learn more about causal relationships between controlled perturbations (treatments) and field observations. Specifically, we seek to design experiments which address the following experimental objective:

> To study in the upper rocky intertidal community the effects on species abundance and distribution of various amounts and compositions of petroleum substances which may disturb the natural system in the following ways:

- physical/chemical disturbance
- direct competition for space
- disruption of competition and predation
- interaction with natural physical disturbances such as wave exposure and dessication

A principal outcome of our study is a set of ecological and statistical design criteria to guide the translation of this overall objective into specific experimental designs.

In attempting to develop specific design criteria we have sought to clarify the role of experimental design theory in this process of design as applied to intertidal ecosystems research. Three pointed questions about experimental design to which we offer answers are:

- Is quantative experimental design theory really applicable to the conditions found in the intertidal environment?
- If so, what standards must be met for an experiment to be statistically valid?
- How much effort (or cost) must the research spend to verify that a particular ecological impact is statistically significant?

APPROACH

The design criteria we identify emerge from a synthesis of information and analysis from several sources. We have reviewed a wide spectrum of literature on rocky intertidal ecosystems, field experiments in rocky shores and laboratory and field studies on the effects of oil in marine environments. We have adopted a functionally-oriented approach to ecological experimentation as described by Dayton (1971, 1975), Paine (1974) and others. We find that the basic assumptions of experimental design are quite compatible with this functionally-oriented approach to ecological experimentation. In both cases, the primary goal is to learn more about causal relationships between controlled pertubations and field observations. Since these relationships are seldom obvious in marine experiments, considerable care must be taken to insure that experimental hypotheses and conclusions can be supported by available data. A number of statistical and probabilistic techniques have been developed over the years to deal with such problems. In our work we have examined some of the most commonly used techniques and assess their capabilities and limitations for petroleum impact studies.

Statistical methods for experimental design and analysis deal with the problem of uncertainty by conceptually partitioning field observations into several components. Generally, these components fall into two categories:

1. Experimental effects due to controlled perturbations or known environmental influences.
2. Residual errors due to extraneous disturbances, sampling uncertainties and other unaccountable sources of variability.

Observation partitioning is most conveniently described with an experimental model which explicitly relates treatment effects and residual errors to observations. As might be expected, the investigator's ability to properly interpret the results of his experiment depends on the relative importance of the residual error component.

If the overall purpose of an experiment is to make reliable decisions about the nature of treatment effects, it is reasonable to measure experimental performance in terms of decision probabilities. In particular, it is convenient to define an optimal experimental design as one which maximizes the probability of making correct decisions about the effects of experimental treatments for a given expenditure of sampling effort. Experimental strategies such as randomization, blocking, fractional replication, and confounding are intended to provide a better allocation of sampling effort and so lead to more nearly optimal designs. The effectiveness and cost of these strategies can be objectively evaluated and used not only to guide the researcher in his search for a good practical design, but also in his assessment of the overall feasibility of the experiment.

The concepts outlined above form the basis for our approach to experimental design. They can be briefly summarized as follows:

1. Experimental models provide a convenient way to partition field observations into true treatment effects and uncertain residual errors. The detailed structure of an experimental model determines the particular method selected for allocating treatments to available experimental units.

2. True treatment effects may be estimated from field measurements even when residual errors are significant. The accuracy of the estimation procedure is influenced by both experimental design factors and uncontrollable sources of uncertainty.

3. The overall merit of an experimental design can be evaluated by comparing statistical performance (i.e., the probability of detecting significant effects) to cost. Optimal designs provide the best performance for a given cost.

RESULTS

In this section we summarize the key results presented in our full report (Moore and McLaughlin, 1977). To facilitate the presentation we identify five principal outcomes and discuss each separately using simple illustrative examples where appropriate. Many details, elaborations, examples and other less important outcomes are presented in the full report.

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CHOOSE A SMALL, MANIPULATABLE POPULATION/COMMUNITY SUBSYSTEM OF THE ROCKY SHORE HABITAT, WHICH IS OBSERVABLE AND CONTROLLABLE

Subsystem size is measurable taxonomically (number of species included) and spatially. Experiments should be conducted on a scale such that treatments and manipulations are under the investigator's control. If experimental, untreated "controls" cannot be established, the experiment should not be undertaken. The number of species included in the experiment should be consistent with the objectives of the experiment, as well as available resources of money and time. Complete taxonomic enumeration is not likely to be necessary. The choice of taxonomic complexity is a highly subjective and diffuclt decision (Paine, 1974). Focusing interest on species guilds can facilitate this decision.

The major disadvantage of reducing the size of the experimental system is that generalizations to larger systems are necessarily restricted. Therefore, the rate of information return from the experiments is reduced. As understanding of the processes governing the system increases, larger subsystems can be defined for experimentation.

It is important to note that we are referring to subsystems at the population and community level. The large variability characteristic of field situations prevents the biological and chemical control necessary for elucidating cellular mechanisms such as associated with biotransformations of petroleum hydrocarbons. Monitoring hydrocarbon levels in tissue of organisms collected in the field should be part of experimental designs. However, cause-effect relationships at this level of biological organization are properly the subject of laboratory studies. As Connell (1973) argues:

> "If questions are asked at the individual or lower levels,...then laboratory experiments are completely appropriate. It is only when the question concerns the behavior of the population in nature,... that laboratory experiments are less useful than field experiments." (p. 50)

Following Connell's discussion we believe that the primary focus of experimental studies of oil in the field should be on population and community effects. Of course, such experiments may require measurement of organism and suborganism variables, but hypotheses to be tested should concern patterns of species abundance and distribution. Understanding foodweb transfers will ultimately require field studies, but in our opinion, the current literature makes clear that too little is understood at sub-organism level to expect field experiments to yield significant information about causal relationships governing rates and routes of hydrocarbon transfer.

The following example illustrates the kind of subsystem we are describing. Consider a protected outer Northern Pacific rocky shore of largely massive, more or less coherent, rock formations. In this environment we expect to find a community of organisms dynamically interacting, heterogenously distributed over space, exhibiting classical vertical and horizontal zonation. Within limited spatial boundaries we expect to find species "guilds" exploiting a common resource, such as rock substrate space; and other guilds exploiting (preying on) the guild below it in the food web.

We choose for a focus of interest the barnacle-mussel zone, typically occurring within the tidal region of 0-8 ft. above mean lower low water (MLLW). Following Dayton's (1971) description we assume that the principal sources of physical disturbance are wave exposure and desiccation. We assume there is no significant log battering and that variations in temperature and salinity do not affect the community.

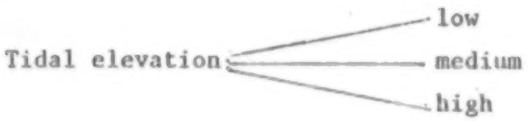
The principal sessile species occuring in this area are various algae, three barnacles--Balanus glandulus, B. cariosus, and Chthamalus dalli, and the mussel Mytilus californianus. Several species of limpets, Thais and Pisaster comprise the important mobile species.

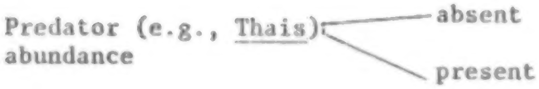
The important biological interactions which are functioning in the community are competition for "primary" space among sessile species and predation by limpets, Thais and Pisaster. Dayton (1971) describes these interactions in detail for the kind of rocky shore we have in mind. The limpet guild plays a particularly important role because it can efficiently graze settling algae and sporelings, as well as prey on and dislodge immature and young barnacles. Experimental evidence such as Dayton's demonstrates the importance of physical and biological disturbances in rocky shore communities. The interplay of dynamic processes--physical disturbance, biological interaction and settlement/recruitment--result in a dynamic, highly variable system which exhibit significant spatial patchiness, as well as large temporal fluctuations.

We further restrict ourselves to the algae-barnacle-limpet interactions as a focal point. The relatively large amount of experimental experience with this sytem reported in the literature is evidence that this is a subsystem which is observable and controllable. In addition, it is noteworthy that herbivore-algal disturbances are often observed following oil spills.

THE DESIGN OF AN EXPERIMENT SHOULD BEGIN WITH A CAREFUL ENUMERATION OF THE DEPENDENT BIOLOGICAL AND INDEPENDENT VARIABLES TO BE CONSIDERED

It is useful, at the outset, to make a list of all the environmental factors (physical, chemical, and biological) which could affect the dependent biological variables of primary interest. These factors should be grouped into classified, concomitant, and nuisance categories. Each category is handled somewhat differently, both in the experimental model and in the field. Classified experimental factors are usually variables of direct ecological interest, including those required to properly describe experimental manipulations. The term "classified" refers to the fact that the factors are discretized into a small number of distinct levels which provide a qualitative measure of magnitude. Two typical examples are illustrated below with some possible classifications:





Environmental factors that appear to be closely related to dependent biological variables but are not likely to be affected by classified factor treatments are good candidates for concomitant variables. A typical example of a variable which meets these requirements in petroleum perturbation studies is species abundance prior to the application of petroleum. Such concomitant variables are accounted for in the linear model as regression terms weighted by unknown coefficients. Analysis of covariance (ANCOVA) is the principal statistical tool available for testing hypotheses when concomitant variables appear in the experimental model (Scheffe, 1959).

When all the experimental factors, concomitant variables, nuisance variables, and factor levels have been defined, the designer should construct an experimental model which specifies how these variables affect the dependent biological observations of primary interest. Generally, they can be partitioned in the following way:

$$\underbrace{y}_{\text{measurement vector}} = \underbrace{\mu}_{\text{grand mean}} + \underbrace{X_m\alpha}_{\text{main effects}} + \underbrace{X_I\beta}_{\text{interactions}} + \underbrace{X_C\gamma}_{\text{regression terms}} + \underbrace{X_B b}_{\text{block effects}} + \underbrace{e}_{\text{residual error}}$$

where μ,α,β,γ, and b are unknown effects parameters and x_m, X_I, X_C, and X_B are known matrices of coefficients. In most applications, the experimenter is primarily interested in the significance of the main effects and perhaps some of the lower order interactions. The regression terms and block effects are included to reduce residual error and are usually not important in themselves.

Higher order interactions arising in factorial models can generally be grouped into two categories:

1. Interactions that are probably significant but are of not particular interest biologically

2. Interactions that are probably not significant.

Insignificant interactions can be incorporated into the residual error term and deleted from the model. Significant interactions which are of no particular interest biologically cannot be ignored in this way but must be treated in much the same way as nuisance variables. These interactions, like block effects, are accounted for in the model but are rarely tested for significance. In principle, then, little will be lost if they are confounded or otherwise lumped together in an incomplete block design.

The number of experimental factors and number of levels defined for each factor should be kept as small as possible. Generally, better performance (higher F-test power) is obtained if samples are used for replication rather than to provide more detailed factor resolution.

We believe the above considerations clearly demonstrate the need for informed biological judgment at nearly every step of the design process. Statistical methods provide tools for evaluating decisions and experimental alternatives but they do not provide the creative insight needed to arrive at useful models of hypotheses. So long as this is appreciated, the ecological and statistical aspects of experiment design can be kept in proper perspective.

FORMULATE CLEAR, CONCISE, EXPERIMENTAL OBJECTIVES AND SPECIFIC TESTABLE HYPOTHESES ABOUT PARTICULAR SPECIES, RELATIONSHIPS AND TREATMENTS

A key step in the design process is to formulate specific experimental hypotheses. Because the effect of oil must be differentiated from natural fluctuations and variability in species abundance and distribution, we must first gain some understanding of these natural patterns and their causes. Given an understanding of the underlying community structure we are then in a position to design experiments to assess the effects of oil. The first step in the oil-perturbation experiments is to determine the extent and magnitude of oil related effects, as a function of the characteristics of applied oil. Subsequent experiments can be designed to elucidate the causal mechanism by which oil affects community structure and the interrelationship between oil and other disturbances. Throughout the design of any experiments on oil effects it is useful to differentiate between initial impact and recovery processes.

The foregoing paragraph outlines several distinct experimental objectives, each of which can lead to specific designs. For purposes of discussion we label experiments intended to confirm our understanding of natural community structure as "initial experiments." The next experimental stage we call "initial impact experiments." These experiments are intended to determine whether or not responses to oil perturbations can be measured. "Recovery experiments" follow initial impact experiments and are intended to elucidate causes of longer-term changes in community structure.

It is apparent that results of initial experiments may determine the nature of subsequent investigations. We therefore recommend a cautious, iterative, incremental approach because of the highly complex nature of the system and the tenuousness of existing models of community structure. The sequential approach to experimentation allows the researcher to identify natural processes acting at the experimental site before perturbations are applied. It also gives him a chance to refine uncertain experimental models, estimate residual error variance, and test field methodology.

To illustrate the specification of clearly stated testable hypotheses, we consider the design of initial experiments for the example rocky shore site described previously.

Results reported by Dayton (1971) and others demonstrate that the pattern of abundance and distribution of sessile species in the barnacle-mussel zone of rocky intertidal habitats is governed by certain physical disturbances and the dominant competitors and predators in the community. In order to confirm this hypothesis for the particular areas in which our oil experiments are conducted, we focus attention on competition among algal and barnacle species and the role of tidal elevation, limpets and *Thais* in determining the recruitment and survival of these sessile species.

We can state this objective in terms of a specific hypothesis:

> *There is no significant change in the abundance of algal and barnacle species occurring in experimental units due to the following factors and/or their interactions.*

- *differences in tidal elevation*
- *presence or absence of limpets*
- *presence or absence of Thais*

Treatments for this experiment consist of different combinations of the levels of the three factors: tidal elevation, limpet abundance and *Thais* abundance. For our example, three levels of tidal elevation may adequately represent the experimental site. We limit the abundance to two levels--present and absent.

LAY OUT EXPERIMENTAL UNITS AND BLOCKS IN SUCH A WAY AS TO MINIMIZE THE RESIDUAL ERROR

The initial steps of the experimental design process define the treatments, nuisance variables and interactions which form the basis for sampling designs. We can now consider in more detail how sampling units should be laid out in the field and how experimental treatments should be allocated to these units. The following points summarize the key issues regarding sample and treatment allocation. Underlying these points is the single concept of minimizing residual error.

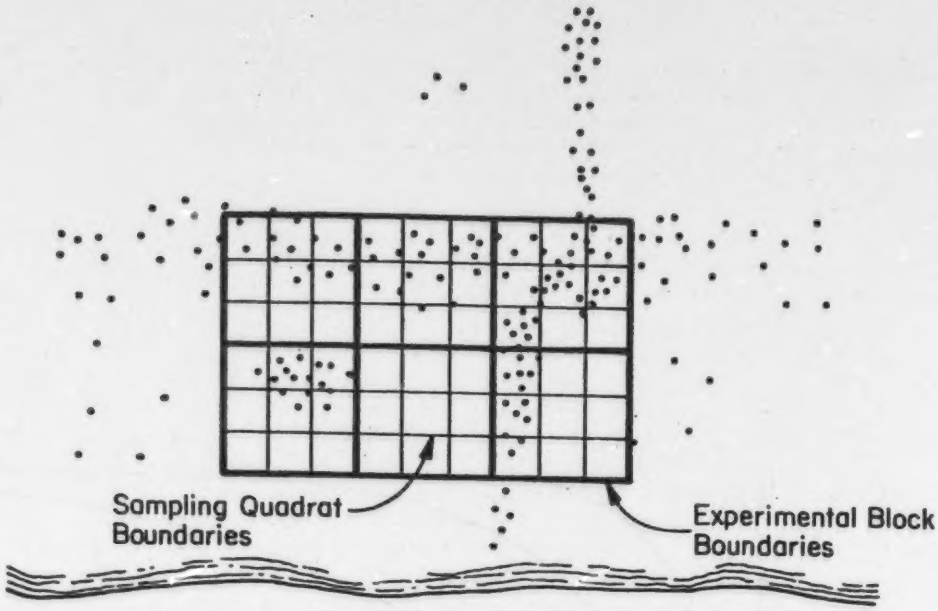
1. Experimental units and randomized blocks should not be laid out in rectangular grids or other systematic patterns if these patterns ignore obvious non-uniformities at the experimental site. Randomized blocks should be positioned and sized to minimize nuisance variations within blocks and maximize nuisance variations between blocks.

To illustrate, consider a barnacle population dispersed over a rocky intertidal site (each dot represents 100 individuals) as shown in Figure 1. The patchiness exhibited by this barnacle distribution is a frequently observed phenomenon due, in part, to spatial variations in wave exposure, substrate composition and other nuisance factors. If a regular sampling grid is super-imposed on the patchy barnacle distribution, abundance counts will vary greatly from quadrat to quadrat within each block (see Figure 1). The experimental model should explain some of this variation but, unless it accounts explicitly for all nuisance effects, its residual error will probably still be very large. One solution to this dilemma is to treat initial (pretreatment) abundance as a concomitant variables. Another solution which avoids the need to assume a regression relationship or perform an analysis of covariance is to simply block sampling quadrants according to their initial abundance. In this case, quadrats of high, medium and low density are grouped into reasonably uniform blocks which reflect obvious terrain and exposure features (see Figure 1b). Density variations within blocks are minimized and those between blocks maximized, as required by randomization theory.

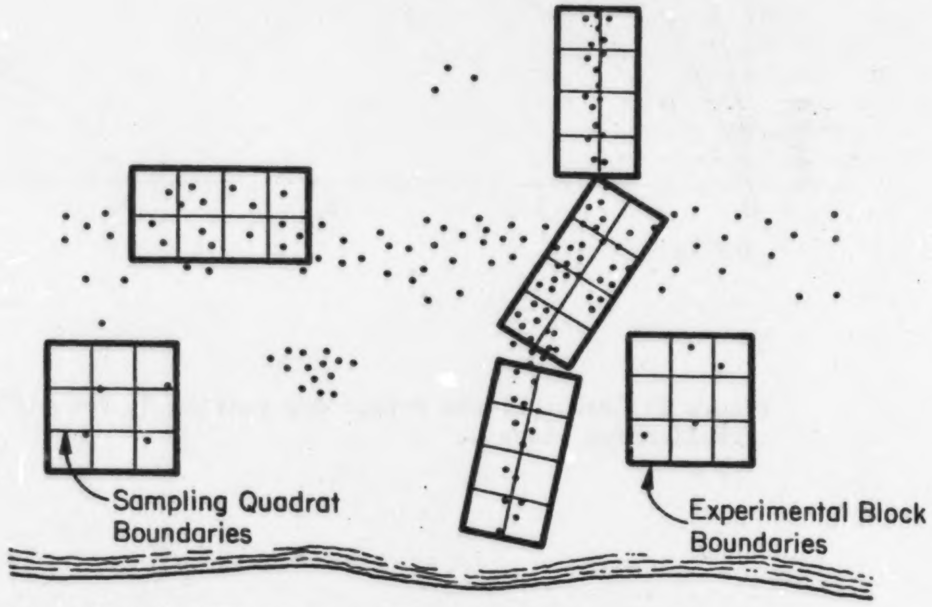
2. The treatment allocation techniques best suited for most (though not necessarily all) intertidal ecological studies are those which rely on randomized block designs. These may be divided into complete block and incomplete block procedures.

3. Complete block layouts are relatively easy to analyze and provide information on all effects included in the experimental model. Unfortunately, they require undesirably large blocks and sample sizes in experiments with more than 3 or 4 factors.

4. Incomplete block layouts based on confounding can reduce block sizes but do not have any effect on the total number of samples required in the experiment. Wherever possible, treatment effects should only be partially confounded (an approach which requires several replicates) so that some information is available on all interaction terms in the experimental model. Generally, confounding is most useful in smaller factorial experiments where high-order interactions are of little interest or in combination with fractional replication in large factorial



a) Regular Sampling Grid



b) Sampling Grid Blocked According to Initial Abundance

Figure 1. Hypothetical barnacle distribution with two alternative sampling grids. Each dot represents 100 individuals.

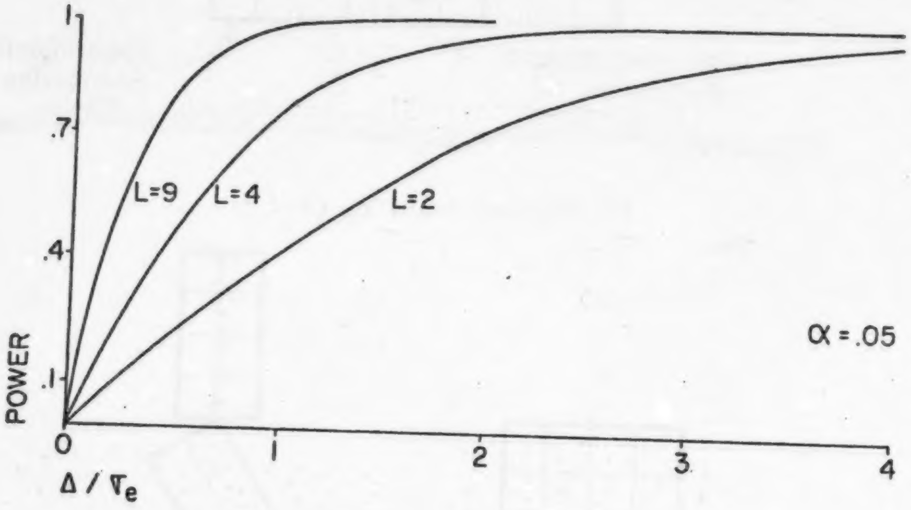


Figure 2. Power of the F-test for testing H_B for different replication sizes L.

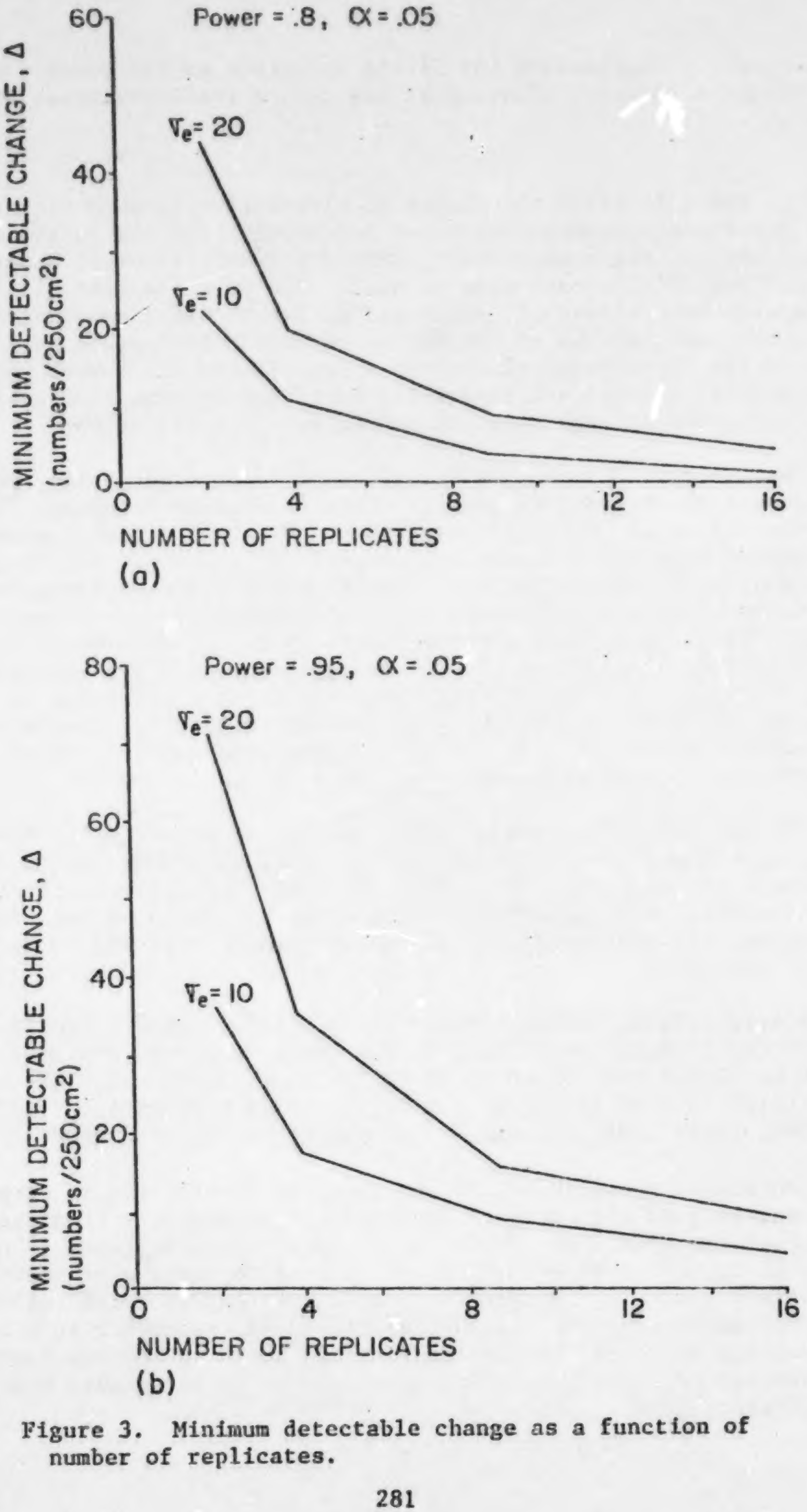


Figure 3. Minimum detectable change as a function of number of replicates.

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experiments. Confounding has little influence on the power curves of unconfounded effects, although it may reduce residual errors significantly.

We can illustrate the choice of alternative layouts with the "initial experiment" example described previously. In the jargon of experimental design, the experiment is called a 3x2x2 factorial experiment. Several possible layouts come to mind. One is a complete block factorial design with blocks of twelve units; another is a confounded factorial design with blocks of six units. A third alternative is to consider each of the three tidal elevations independently and layout three 2x2 experiments, one at each elevation; or to use a split-plot design, separating blocks into plots according to tidal elevation.

We can rule out this latter alternative because we are specifically interested in main and interaction tidal elevation effects. These are excluded if we use three 2x2 experiments or a split plot layout. Confounded designs are suitable for cases in which we can expect experimental error to be reduced by using blocks which are more homogeneous, or which can be subjected to more uniform techniques, than a complete block. These conditions are not likely to be encountered in this case. The principal expected source of heterogeneity is tidal elevation, which is already included as a factor. Variability over clusters of two or four 250 cm^2 units, is unlikely to be reduced by using blocks of one or two units. Where a larger number of units comprise a block, confounded layouts are of more interest.

We are left then with a 3x2x2 complete block factorial design in which each block contains twelve units, each unit receiving a different treatment corresponding to each of the twelve possible factor-level combinations. Allocation of treatments to the units in any block should be random. If replicate blocks are used, these also should be positioned randomly.

THE FEASIBILITY OF AN EXPERIMENT (ITS ABILITY TO DETECT SIGNIFICANT ECOLOGICAL IMPACTS FOR A GIVEN EXPENDITURE) IS DETERMINED BY 1) THE LEVEL OF EFFECT CONSIDERED TO BE SIGNIFICANT, 2) THE RISK THE RESEARCHER IS WILLING TO TAKE IN MAKING A DECISION ABOUT SIGNIFICANCE, 3) THE RESIDUAL ERROR VARIANCE, AND 4) THE MAXIMUM ACCEPTABLE SAMPLE SIZE.

An accurate assessment of experimental feasibility is very difficult unless good estimates of experimental variability (residual error variance) are available. When the residual error variance is not well known it is useful to examine the region of feasible experiments defined by minimum detectable effect vs. sample size curves drawn for different residual error variance levels (see Figure 4). Whenever such relative evaluations are made, the designer should check to see how feasibility is affected by rather arbitrary restrictions on acceptable test power or significance level.

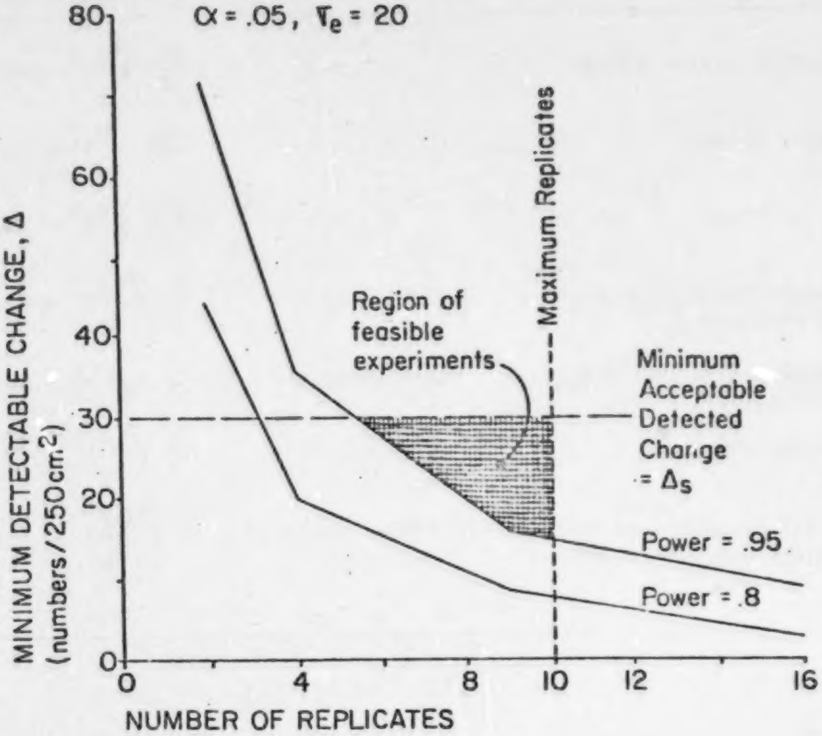

Figure 4. Minimum detectable change versus number of replicates and region of feasible experiments for example in text.

Table 1

ANOVA Parameters for Complete Block 3x2x2 Factorial Layout with L Replications of Each Treatment

Hypothesis	Degrees of Freedom	Non-Centrality Parameter
H_A: Tidal Elevation Effect	I-1 = 2	$\frac{JKL}{2} (\frac{\Delta_A}{\sigma_e})^2 = 2L (\frac{\Delta_A}{\sigma_e})^2$
H_B: Limpet Effect	J-1 = 1	$\frac{IKL}{2} (\frac{\Delta_B}{\sigma_e})^2 = 3L (\frac{\Delta_B}{\sigma_e})^2$
H_C: *Thais* Effect	K-1 = 1	$\frac{IJL}{2} (\frac{\Delta_C}{\sigma_e})^2 = 3L (\frac{\Delta_C}{\sigma_e})^2$
H_{AB}: Tidal elevation--Limpet Interaction	(I-1)(J-1) = 2	$\frac{KL}{2} (\frac{\Delta_{AB}}{\sigma_e})^2 = L (\frac{\Delta_{AB}}{\sigma_E})^2$
H_{AC}: Tidal elevation--*Thais* Interaction	(I-1)(K-1) = 2	$\frac{JK}{2} (\frac{\Delta_{AC}}{\sigma_e})^2 = L (\frac{\Delta_{AC}}{\sigma_e})^2$
H_{BC}: Limpet--*Thais*	(J-1)(K-1) = 1	$\frac{IL}{2} (\frac{\Delta_{BC}}{\sigma_e})^2 = \frac{3L}{2} (\frac{\Delta_{BC}}{\sigma_e})^2$
H_{ABC}: Tidal elevation--Limpet--*Thais* Interaction Effect	(I-1)(J-1)(K-1)=2	$\frac{L}{2} (\frac{\Delta_{ABC}}{\sigma_e})^2 = \frac{L}{2} (\frac{\Delta_{ABC}}{\sigma_e})^2$
Residual Error	IJK(L-1) = 12(L-1)	
Grand Mean	1	
Total Number of Units	N = IJKL = 12L	

We can illustrate these notions of experimental feasibility by further developing the "initial experiment" example we have been describing. Several preliminary analytical steps precede the evaluation of feasibility.

First, we form an abbreviated ANOVA table for the 3x2x2 design, showing the degrees of freedom and the F-distribution non-centrality parameter λ (Table 1).

Analysis of the power of the F-test for testing the significance of the hypotheses shown in Table 1 allows us to determine the number of replicates necessary to have a given probability of obtaining a significant result. For example, Figure 2 is a graph of the power of the F-test for H_B versus Δ/σ_e for different replication sizes. It is readily apparent from this figure that for 9 or more replications, the probability of detecting a significant effect is relatively high for a wide range of Δ/σ_e values.

Given an estimate of σ_e^2, the variance of the residual error, we can compute estimates of the minimum detectable effect, Δ, for given replication sizes and powers. Recall that the observations are the abundances of various species of algae, barnacles, limpets and Thais. We must estimate σ_e for each of the species to be measured. Estimates are given in Table 2.

Table 2

Estimates of Residual Error Standard Deviation, σ_e for Various Species

Species	σ_e (number/250 cm^2)
Algae	--
Balanus glandula	10-20
Chthamalus dalli	20-40
Balanus cariosus	20-20
Acmaea spp.	--
Thais emarginata	10-30

Figures 3a and 3b show the relationship between minimum detectable change versus number of replicates for various values of σ_e for power of .8 and .95. Note that the minimum detectable change is proportional to σ_e for a given power. Also, note that the minimum detectable change for $\sigma_e = 20$ and power of .8 is virtually the same as that for $\sigma_e = 10$ and power of .95. Or, in other words, for a given number of replicates if we can reduce σ_e from 20 to 10, we increase the power from .8 to .95.

We can redraw Figure 3 for a given σ_e and various values of power. Such a graph is shown in Figure 4. From this display we can obtain some sense of the economic feasibility of the experiment we have designed. We assume that costs are proportional to the number of replicates and, for example, that we have a budget constraint which limits the experiment to a maximum of 10 replications. We also assume that the minimum acceptable detectable change is 30 individuals per 250 cm^2 quadrat and that we want a .95 probability of detecting this change at a .05 significance level. The cross-hatched area in Figure 4 defines the region of feasible experiments to meet these constraints. As shown in the figure, 6-10 replicates of the 3x2x2 experiment are sufficient to provide a .95 probability of detecting changes of at least 30 organisms per 250 cm^2, if such changes exist, at .05 significance level.

Finally, for this experiment we look at the marginal sensitivities of σ_e to L. In Figure 5 we present the relationship between the maximum tolerable σ_e, σ_{ea}, and the required number of replicates L_a, given Δ_s, power and α. For L_a greater than six the relationship is virtually linear, indicating that the marginal gain in the acceptable level of residual error standard deviation is constant for each additional replicate. Given an estimate of the residual error variance and choices for power, α and Δ_s we can readily determine the necessary number of replicates from plots such as Figure 5.

PROBLEMS

We have encountered three critical problems in this study for which we see no clear solution:

1. The apparent residual errors with which we must work in rocky intertidal ecosystems are quite large relative to the magnitude of differences (changes) we want to detect. As a result, large numbers of replicates are needed to obtain statistically acceptable designs. However, such large number of replicates may lead to economically infeasible experiments. To the extent that the residual error is a function of inadequate knowledge and poor models, the problem can be alleviated by further research which leads to better (more certain) explanations of observed phenomena. However, much of the variability we observe may be an inherent quality of the systems we are studying; in which case we have little recourse.

2. Petroleum substances are complex chemical mixtures, which have several characteristics that complicate the field experiment problem. First, organisms have not evolved in the presence of oil in the amounts, compositions, and distributions of it associated with spills. Oil is for practical purposes a foreign substance in rocky shores. Unlike manipulation of, say, predator density, application of oil is a disturbance for which the community has no historical precedent. This fact should not validate properly designed experiments. However, choice of

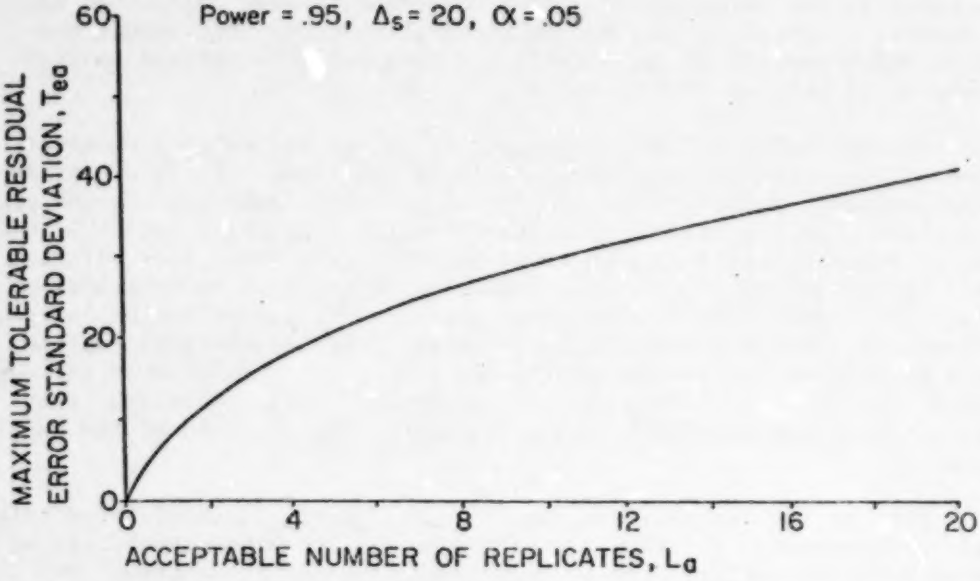

Figure 5. Marginal sensitivity of σ_{ea} to the number of replicates, L.

dependent variables, experimental treatments, observations and unit and interpretation of results may be more difficult because we have relatively little definitive knowledge with which to develop testable hypotheses.

Secondly, oil is pervasive, not selective in its effects. Oil directly and simultaneously may affect physical variables, (e.g., substrate area), the chemical environment, and the biological environment (e.g., increase/mortality). Because of the pervasiveness of oil effects, designing experiments which allow particular relationships to be investigated appears to be extremely difficult. Oil cannot be indiscriminantly applied and expected to exhibit only selective effects of interest to the investigator. Even when experimental objectives are carefully selected, it may not be possible to devise treatments and units which confine oil physically and chemically to desired spatial units or to only species within a given unit.

Another difficult characteristic of oil is its complex chemical composition. Oil is not a single uniform substance. It is a mixture of many different hydrocarbon compounds of different molecular weight and structure. As a result of different chemical properties (solubility, vapor pressure, etc.) each compound has different biological effects. Oils from different origins are different mixtures of hydrocarbons so their biological effects also vary. In order to assess the biological effects of oil and compare different oils, detailed chemical analyses must be obtained. An experiment using oil as a treatment must include a measurement of hydrocarbon composition by type (e.g., paraffin, aromatic, etc.) and molecular weight throughout the duration of the experiment.

Part of the chemical complexity of oil is its instability in natural environments. The volatility, solubility and biodegradability of lower molecular weight hydrocarbons leads to rapid "weathering" of spilled oil, i.e., rapid changes in the hydrocarbon composition of the spilled material. As a result, an oil "treatment" is not constant; it is time-varying, further complicating the experimental problem. Again, carefully detailed chemical analyses are a necessary part of any investigation of the biological effects of oil. Chemical data throughout the course of an experiment is the only way of accurately describing the kind and amounts of chemicals to which organisms are exposed.

3. True experimental "controls" do not exist in field experiment situations. At this point we have little more to say about this problem than has been said by previous authors (e.g., Connell, 1973). There seems to be no solution to this problem. Two questions to which we seek answers are: What is the validity of comparing replicated samples from two "similar" sites, one treated and one control? What are the statistical and experimental trade-offs between multiple sites with few sample replicates at each site and one control site with many replicates?

CLOSING REMARKS

It is instructive to review the three questions posed at the beginning of the paper. We will attempt to give brief answers to each question based on our study.

- Is quantitative experimental design theory really applicable to the conditions found in the intertidal environment?

Yes, provided that the designer properly accounts for the patchiness, zonation, and complexity inherent in intertidal ecosystems. This provision obviously requires a balancing (tradeoff) between statistically feasible objectives and cost. The more complex the system addressed, the higher the cost of significant information.

- If so, what standards must be met for an experiment to be statistically valid?

The experimental layout must be properly randomized so that within-block nuisance variations are minimized and the experimental model must explain a reasonably large fraction of observed sample variability (i.e., the residual error variance should be small relative to the measurement variance). Also, assumptions used to construct response surfaces, delete interactions, or ignore aliases should be ecologically justified. Finally, the experimental model, sampling layout, and field methodology should be consistent with the researcher's intuitive understanding of ecosystem behavior.

- How much effort (or cost) must the researcher spend to verify that a particular ecological impact is statistically significant?

This depends on the level of effect the researcher considers significant, on the risk (probability of error) the researcher is willing to accept in his decisions, and on the level of residual error variability present after the best available experimental model has been fit to the data. Systematic techniques are available for relating all of these design specifications to sample size (or cost) for a given experimental layout.

Finally, we offer our primary recommendation for further work. In our study we have addressed the generic problem of experimental design in some depth and detail. We have not yet, however, identified the number's of blocks, units, samples, replicates, controls which are likely to be required to detect a 10%, 50%, 100%, 500% change from any source in the abundance of, for example, *Thais*, *Mytilus*, *Balanus*, *Fucus*, *Pisaster*, etc. at a confidence level of 0.75, 0.90, 0.95. Such an analysis should be performed on data sets currently available from the Pacific N.W. or Alaska intertidal zones.

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PART III

SELECTED DISCUSSION TOPICS
AND
SYNOPSIS OF REVIEWERS' COMMENTS

18. DISCUSSION ON CHEMICAL ANALYTICAL REQUIREMENTS AND INTERCALIBRATION

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I will review very quickly what the field chemists, if you will, have done in the area of intercalibration and quality control and see how that may be applied to the chemistry in the effects program.

First, the chemists in the field analytical program for hydrocarbons had two problems with which they had to contend. The first was that hydrocarbon concentrations are extremely low so very sensitive methods had to be established right from the beginning. Second, was the problem of finding petroleum type hydrocarbons, which could be ascribed with some confidence to petroleum, in the background biogenic hydrocarbon mixture. Methods not only had to be sensitive but had to be very highly discriminatory. So our methodology evolved from the initial packed column gas chromatography to a system such as Bill MacLeod described earlier -- the glass capillary gas chromatography coupled to a mass spectrometer with a data system with spectral library matching and gas chromatography capability. I think at the present time every BLM field program has one of these complicated pieces of equipment available to the program.

In addition to evolving instrumentation, there was also a good bit of effort being put into the sample extraction, cleanup and analysis steps. Along with those efforts, the National Bureau of Standards operating with funding from OCSEAP and BLM has distributed three sets of hydrocarbon-containing material on a national basis to serve as intercalibration samples. The first of these were two sediment samples from the Alaska region, one from Hinchinbrook Entrance and another from the Katalla River area. These two sediments (distributed approximately two years ago) had total hydrocarbon concentrations in the ten to one hundred parts per million range. Approximately a dozen laboratories analyzed these two samples and the results were recorded and rather enlightening. The range of concentrations found was approximately two orders of magnitude, so our estimates of environmental levels at that time were very unprecise. But with this sort of information, it was decided that the next round of intercalibration should not be on a low level sample, but to make it easier on ourselves, on a high level sample. Next, Ian Kaplan of UCLA volunteered to collect some sediment from Tanner Basin and to add to it some Southern Louisiana crude oil. When this sample was distributed, lo and behold everybody did find crude oil and the range of the results was only approximately one order of magnitude. So we were making progress. By the way, this is not unusual; people had not yet done any national intercalibrations on this type of analysis. A similar program was begun a few years ago at the national level--for lead, which is very difficult to analyze. When that intercalibration for lead was conducted, the initial numbers varied by at about three orders of magnitude. Once that problem was recognized, the analysts got together and discovered the problems. I think now that there are quite a few laboratories who can analyze lead and have a high degree of confidence in their numbers. So we are approaching that with the hydrocarbon methodology. A third intercalibration sample, this time a tissue (Mytilus) has also been distributed, but to my knowledge, the results have not been forwarded. I believe they are due about January or February of 1978.

One problem with these intercalibration samples is that they have all been one-shot efforts. You have one chance to analyze them and send your results in, and if you don't like what you've got, you can't go back and do it again. So with BLM's blessings and obvious need, we are going to acquire a large volume of sediment for use as an intercalibration reference material. Bill MacLeod at NOAA's National Analytical Facility will characterize this material, and develop some feeling on its degree of homogeneity. Samples will then be distributed to all of the chemists in all of the BLM programs, not just OCSEAP-funded programs. I think it is highly desirable, and should become a matter of contractual obligation if necessary, for the chemists involved in the effects program also to receive the material and analyze it.

This material will be available approximately March 1, 1978. Investigators performing hydrocarbons analysis in the effects program should request this intercalibration material, and I'll make sure you get it. These intercalibration activities are extremely valuable because you can discover problems in your technique that you had no idea were there. Since we are going to have enough material to analyze repeatedly, you should be able to document your improvement in methodology. And it's extremely important to do that in the effects program, because as you've seen yesterday and this morning, we are talking about measuring parts per billion exposures. It becomes extremely critical to document those exposures in increasingly narrow precision bands. I hope that everyone will voluntarily take advantage of the availability of the intercalibration material.

Discussion:

S. D. Rice: Could tissue samples be sent out for calibration? There are a number of labs not working with sediments, and I'm not familiar myself with what the problems are with sediments.

Calder: That's definitely a problem if you are not involved in a routine analysis with sediments. There are two ways to get around that. One is, get good at analyzing sediments, which is not that hard and probably easier than doing tissues. Secondly, you could perhaps impose upon someone else to do the initial extraction of sediments, and then you take it from there and it would be identical with tissues. As far as getting tissue samples for distribution, that's not currently planned, but could be done.

Question: What were the criteria for the selection of the two places that you mentioned, where sediments were collected? You mentioned Hinchinbrook Engrance and the Katalla River--the old oil site.

Calder: Right, there was a history of oil seepage at the Katalla River Site--that is why it was chosen. Hinchinbrook was perhaps a more pristine sediment. It was soon quite obvious that we weren't ready to tackle sediments at that low level and do a good job of it, so we then went to Tanner Basin and I think that sample will be somewhere in between the earlier extremes.

Calder: Let me add two other things then we'll get some more questions here. The National Bureau of Standards is still working to develop a certified hydrocarbon reference material. I am sure that it will be at least two years, and probably longer than that, before it is available. So our sediment program is noping to fill at least this two-year gap. The second point is that in terms of evaluating laboratory procedures, separation and cleanup, the BLM also distributed not too many months ago a synthetic hydrocarbon mixture with known composition and concentration which could be used in a manner to check separation, recovery efficiency and so on. That particular sample has been exhausted, but anyone could make up his own synthetic hydrocarbon mixture of known composition and concentration. If there were a demand for it, another large volume of synthetic mixture could probably be prepared and distributed somewhat more widely than before.

Wolfe: I might mention that NBS is on the verge of releasing a standard mixture of hydrocarbons adsorbed on glass beads through which water is passed, producing a solution which is guaranteed by NBS to have a constant and predictable concentration. This resultant solution can then be used as a standard reference solution at relatively low concentrations. That should be available I think right now.

Scott Warner: You might mention that in terms of getting better interlaboratory continuity of data, BLM at least for the time being is giving up the idea of standardizing a method but instead is relying upon performance criteria. It's perhaps not as good as having an unlimited supply of calibration sediment, but at least it is something that we are going to do now and check out how well it works.

Tom Church: Those who are doing the metal analysis, or metal in petroleum, can use numerous standards that are now available for metals. One of the more extensive ones is a set of marine samples circulated by IAEA in Monaco, including a whole suite of biological organism samples. Experience shows that there are really two stages in a calibration exercise which should be separated. One is the instrumental stage which is a simple circulation of the known standard for which people have to qualify and quantify their recovery and instrumental procedures. And then there is the preparation stage which is where you must circulate the sample material. The first should precede the second. This allows one to isolate the problem in the techniques used to process the material, i.e., contamination that stays and is not an instrumental problem.

Calder: For all these reasons, it is very important for the chemists involved to demonstrate the level of confidence that they and others can place on their different techniques. So again I encourage you to take advantage of the reference sediment samples and any other method that can be used to cross-check your procedure with other laboratories.

19. FUTURE RESEARCH NEEDS

- Discussion -

To stimulate discussion, a questionnaire was developed and distributed in advance of the Review. Many of the participants in the Review completed the questionnaire (or parts of it) and these responses entered into the morning's discussion. The questionnaire is reproduced below, followed by the transcript of the ensuing discussion.

Section I - Single Species Bioassay and Toxicity Testing

1. With respect to Alaskan OCS oil and gas development, are additional acute toxicity bioassays needed on Alaskan marine organisms? Why?

2. What toxicants, exposure techniques, analytical methods, or other experimental conditions not previously used should be tested in these acute bioassays?

3. What 5 Alaskan marine species or life stages are most in need of study (i.e., acute toxicity bioassay)? Why?

4. What specific effects or processes should be examined under low-level chronic exposure conditions?

5. What are your recommendations for test organisms for sublethal effects studies? Why?

6. How can the results of acute and chronic bioassays be made more useful to policy makers and regulatory agencies, in terms of experimental design or data presentation?

Section II - Population, Community and Ecological Effects

7. What is the best approach for estimating population-level and ecological effects of oil exposure in marine systems?

   a. Direct experimental work in intentional, controlled spill situations.

   b. Observations after accidental spills.

   c. Petroleum perturbations in contained ecosystems (i.e., microcosms, tanks, walled-in ponds, etc.).

   d. Computer modeling of ecosystem dynamics and effects results.

   e. All or none of the above. Other?

8. a. What primary science objectives should be addressed through intentional oil spill experiments in the marine environment?

   b. What technical problems would you expect with the above objectives? How would you design the experiment to ensure success?

   c. What alternative approaches (to intentional spills) are available for addressing the above objectives, and what are their limitations?

9. The OCSEAP SOR Team is currently responding to accidental oil spills to obtain data on physical and chemical behavior of oil in marine systems. If logistic and financial support were available, would you have specific research objectives which should be addressed of an accidental spill situation?

   a. What are these objectives?

   b. Under what spill conditions (weather, oil type, locations, water depth, etc.) would these objectives be feasible?

   c. What alternative approaches (other than spill circumstances) are suited to these objectives? What are their limitations?

   d. What ancillary measurements should be performed simultaneously at the spill to enhance the value of your suggested study?

Section III - Chemistry and Intercalibration

10. Hydrocarbons may be measured by a variety of analytical techniques, gravimetric, IR, UV, UVF, GC, LC, CC-MS. Under what conditions are the simpler measurements (IR, UV) satisfactory? When are the more sophisticated methods required?

    Should non-hydrocarbons be routinely assayed in effects experiments? If so, how?

    Even when a single hydrocarbon is used as the best toxicant, should cleanup and separation techniques be used before analysis?

11. What (if any) uniform procedures be adopted for the following:

    a. Hydrocarbon analytical methods for field and experimental samples (water, tissues, and sediments).

    b. Method of preparation of water soluble fractions or of oil sediment or food.

    c. Method of introduction of oil/hydrocarbon to the test system.

Section IV - Future Directions

12. What aspects of the OCSEAP effects program should receive increased emphasis next year? Decreased emphasis? Why?

13. What improvements can you suggest in the review approach taken at this meeting? How frequently should reviews occur? Should the proceedings be published? Should the review be expanded to include investigators from the disciplinary areas of the program?

Wolfe: Good morning. Our agenda calls for a discussion of research needs and directions at this point, and I'd like to start by reviewing the discussion we had at the restaurant last night. We went through the twelve questionnaires that had been completed and looked at the responses to question three which was, "What five Alaskan marine species or life stages are most in need of study?" As said last night there were relatively few specific recommendations. The recommendations that came in included broad statements like: we should do representative species of representative groups from everywhere in Alaska. That is the kind of statement that us bureaucrats have become professional at writing and putting into TDP's. We did though, get a couple of specific recommendations and among those that perhaps should be considered is Tom Church's recommendation. He suggested that there may be key trophic elements in the system that should be looked at very specifically based upon either field experimental results or field ecology trophic study efforts. The specific organisms that he pointed out were the flagellates which were suggested in the CEPEX experiments as a critical element in the support of the entire system in the CEPEX bags. When the flagellates collapsed there was a sequential collapse of everything else. We didn't get to a discussion of how conclusive that result was, nor to a discussion of what kind of observations really warrant extensive toxicity testing on those organisms. Is anyone familiar with the CEPEX experiments to the extent that they could comment on the flagellates? And their role in that system?

Jack Anderson:

Not really, but the CEPEX bags are a closed system. Can we really expect to relate to that?

Wolfe:

There's a controversial issue! How large or how small can a closed system be and still be representative of the real open system?

Jack Anderson:

Basically what you find is that things happen faster in closed systems. There may be the same sorts of changes taking place, but the tendency is for the phenomena that you see in the real world, i.e., in natural systems, to be intensified in closed systems. I don't really see how that justifies trying to look at toxicity in phytoplankton or zooplankton at all.

Wolfe:

In fact one of the recommendations on one questionnaire was that we should put increased emphasis on toxicity work on microscopic water column organisms especially those that might associate with the surface and might be nearest to a floating oil slick. Does anyone have any thoughts or reactions to how we should be looking at phytoplankton or zooplankton in terms of toxicity?

Dave Peters:

Seems to me it would be worthwhile to work on something that you can culture through an entire life cycle so you can look at toxicity throughout its life. That might be zooplankton.

Byron Morris:

Is it valid to talk about zooplankton as an entity? Aren't zooplankton as different in their responses from species to species as other organisms are? Shouldn't we select which zooplankton are most important or representative in the system?

Bori Olla:

Could we look for animals in the zooplankton or in the intertidal that could be considered Drosophila-like? Do these animals exist? If they do exist, it strikes me as being potentially valuable to try and seek these animals out. Because of their short generation times and potentially relatively easy culture methods, it could be highly highly productive and relieve what I find to be disturbing amounts of energy input from the investigators.

Comment:

If you started down at that low level of the pipe, i.e. with ciliates, you're going to have to look at microbial counts because if you think about the energy inputs effective below the thermocline then what is your primary energy source, what are your inputs of materials? You have to think like that and likewise during the winter months when there isn't enough phytoplankton, what replaces the primary production? So when you think about the energy requirements of flagellates and ciliates, you look at the recent literature especially that from Denmark, you'll recognize the microbes are the main source of food. And so the point is, if you know that chain then you really know the whole thing.

S.D. Rice

It seems like you're mixing up two different objectives here in your discussion: One is what are the most sensitive animals or plants? Another in the case of your Drosophila discussion, is there an animal that you can use in a variety of challenge experiments to look at the effects of temperature or salinity on other types of variables or to determine what are the most toxic components? You're getting away from what are the most sensitive animals. You're mixing up two different discussions.

Ron Atlas:

I'd like to repeat a comment I made last night. That is, I don't think you can just pick a species and do toxicity testing in a flow-through or closed system. I think you've got to look at changes of community structure. If you want to test trophic levels, that's fine, but I don't think you can pick an organism out of each trophic level and say it is representative in terms of toxicity. I think you've got to work with mixed systems and look at what's happening. When you see something you may have to verify it with the single-species-toxicity laboratory test - but I don't think you're getting very far picking a few organisms for toxicity testing in experimental chambers. There are two approaches: If you see an effect within a community structure then you can pick out individual species and see whether there will be a toxic effect on the individual. The other way is if you are in need of determining particular types of responses that you want to observe in the community then you may initially pick out species and determine types of responses within the community situation level.

Jack Anderson:

I'll go along with that.

S. D. Rice:

I would too, but there's only one catch to it. Microbes and phytoplankton seem to be relatively insensitive and are also highly variable. So you need an experimental design, a logistics approach, that will allow you to see effects under environmental conditions.

Jack Anderson:

We have done that of course in our trays where we can have quite a few bivalves present in a single container in the natural environment.

S.D. Rice:

You're really focusing on only one trophic level, detritivores basically. You're only looking at one trophic level in terms of your response.

Jack Anderson:

Well, bacteria are present ...

S. D. Rice:

But you're not looking at the bacteria, you're looking only at the clams.

Ron Atlas:

We've repeated that approach with boxes of invertebrates in a couple of ways. Doug argued with me last night that if we got to higher organisms, patchiness of distribution would preclude that sort of approach. I'm not sure that that's true, I think you can pick areas without needing too many representative boxes.

Wolfe:

It seems clear that if you put out boxes containing a structured community that you introduce into the system then you can guarantee sampling success. If you randomly put recruitment boxes or bricks out on the intertidal zone then I think you've got a different problem.

Ron Atlas:

It depends on whether the observed initial state represents a removal of most of the original community in which case what you're studying is recolonization. If you're looking for an initial toxicity and initial lethality study in which you're worried about wiping out particular selected organisms then patchiness is a problem. But if you really oil an area to the point where organisms either respond by leaving or respond by dying so you're left on day one of your experimental spill situation with areas void of living organisms in that area and on day two you start looking at what migrates back in then I think you can have hope.

Wolfe:

You're suddenly looking at recovery, or recruitment into an unpopulated area. You're looking at big changes in the community. And that may be very valuable.

Usha Varanasi:

I have a question. Are we going to look at any synergistic or antagonistic effects of oil? It seems we've been addressing for the most part the effects of single components in isolated systems.

Wolfe:

How does one go about examining potentially synergistic or antagonistic effects?

Usha Varanasi:

There are two ways, one by doing field experiments and the other by simulation in the laboratory.

Wolfe:

Can one really hope to identify synergistic or antagonistic effects under field circumstances?

Usha Varanasi:

Well if one can identify to some extent in the laboratory the effects of individual components, perhaps it is time to begin to work with more than one component, so we can say that an observed effect resulted from X or Y or from XY.

Don Malins:

The point is not that this is going to be solved in five months but I think we've got to get some idea about whether the studies conducted on the singular systems are in any way realistic. That just seems to me to be the immediate issue. Also perhaps whether we will be able to solve all the simultaneous equations for the synergistic effects. But how important are those effects? We can look at some isolated binary or tertiary systems. But how influential are they likely to be? Very influential, or hardly influential at all? We know practically nothing about that now. I think that's what those who are asking are trying to decide.

Wolfe:

What kind of community or population parameter do you look at to determine how influential a particular single species or single organism effect might be.

Don Malins:

I'm not speaking of that particular issue so much as I'm thinking what is the difference say in contaminant accumulation into families of organisms and how are the effects modified under different environmental condition? Now when you start getting into the issue of the ecosystem the problem is even greater. But I'm not sure that we're going to make any sense out of this by translating it immediately into an ecosystem. I think it has to be examined in small parts to begin with because we don't know anything about it.

S.D Rice:

I don't believe you can effectively study synergisms in natural communities. You have synergisms between environmental factors and contaminants at all levels of the community and synergisms between the different community components, all kinds of secondary effects and complex interactions. It's just too complex to study quantitatively. The net result is that you can end up with the wrong idea of just what the sequence of events was and how to interpret them. So I think to study synergisms, you have to go back to single species and eliminate the community inteactions. I think you have to determine the synergistic effects of different contaminants and environmental variables on several individual species first, and see whether they are additive or synergistic, before going to the community level.

Wolfe:

So the only way to realistically and feasibly look at synergistic effect right now is to use small relatively simple highly replicable systems which can be tested under a multivariate experimental design. Is that what I'm hearing?

Byron Morris:

For ten years now we've been doing single-species dosing type studies, and we've assembled a reasonable track record; ie. lots of people have exchanged results and compared notes, etc. We know there will be large differences between what you find with single-species dosing experiments and what you will always find when you start looking at whole ecosystems, effects will be real and will occur at much lower levels. It's almost 1978, it's time to start thinking at least about how we're going to look at the whole system in terms of effects.

Don Malins:

I think that's a fine idea, but if you do it you're going to have to be the one who's going to have to make this story hang together and if you can do that, that's fine. I think it will depend very much on the sort of experiments outlined. We're talking I assume about synergistic and antagonistic effects and multiple effects, and I personally find that this story is very hard to put together on the basis of experiments thus far.

S.D. Rice:

I think that one reason why we can't is that there is virtually no data on what we've discussed regarding synergistic effects of oil with either environmental effects, with components of oil or with PCB's. We have only limited impressions of what those effects might be as of now.

Tom Church:

The real effect is going to be the net effect in the real world so why are you hung up on whether the single elements will go belly up. The real synergistic experiment is that soon we'll have no fuel and we want at least to start thinking about designing the experiment that pulls these things together.

S.D. Rice:

I agree with you that you end up with the net result, but you won't know how, or why, or what the sequence of events was. And if you don't know that, and you do the single experiment, the single spill experiment, in Cordova or someplace like that, you're not going to have any idea whatsoever of how to extrapolate that to the Beaufort Sea or to Lower Cook Inlet or anyplace else.

Tom Church:

You may not be able to. You may have to do it at each place you're concerned about.

S.D. Rice:

All right, then you're going to have to do it at each season each year, both under poor and good weather conditions. Now you're talking about 100 or some odd spills. And you had better replicate them, you will need to replicate them because you don't know what variables are really important.

Don Malins:

That's right, I think you've got to understand the system and you've got to understand the processes because until your particular system is understood, you have no information at your disposal to make any reasonable valid judgements on what a perturbation might mean to that system.

Dan Costa:

I submit that you advocate some kind of community approach to identify what these single species are going to affect in terms of total structure. Sure you can go out there and do experiments on oil spills and then see before and after effects. First a description of what the ecosystem is, in terms of who's eating who and where's it going and then possible identification of major food items which are important if you knock out that group of organisms. Now that's somewhat a naive approach because it completely excludes predator-prey interactions. But at least it is a start because you can then begin to say what may be your important species instead of just saying Well we'll get an economically important species. At least then you may be starting to understand what the system is and that's not an easy thing to do--just to describe it. If we knew what the system was, then we might be able to set up maybe the important interdisciplinary experiment on that system.

Wolfe:

Maybe now is the time to have a quiz on Feder's Bering Sea food web (Figures 1a and 1b).

Byron Morris:

Is that real or is that a figment of his imagination? I'm only being facetious, but have all those relationships really been established?

Wolfe:

We've invested money into the analysis of a lot of samples to come up with the relationships depicted in that figure. There are currently no numbers on it and I'm sure its still partly a figment of our imagination. However, if what Dan just said has any basis in truth then you have to begin to understand these kinds of relationships in association with discreet cause/effect relationships that become understood independently in experimental systems. Then and only then can we expect to make predictions of what the net effect is on a system at any time after the perturbation.

SOUTHEAST BERING SEA - Food Web

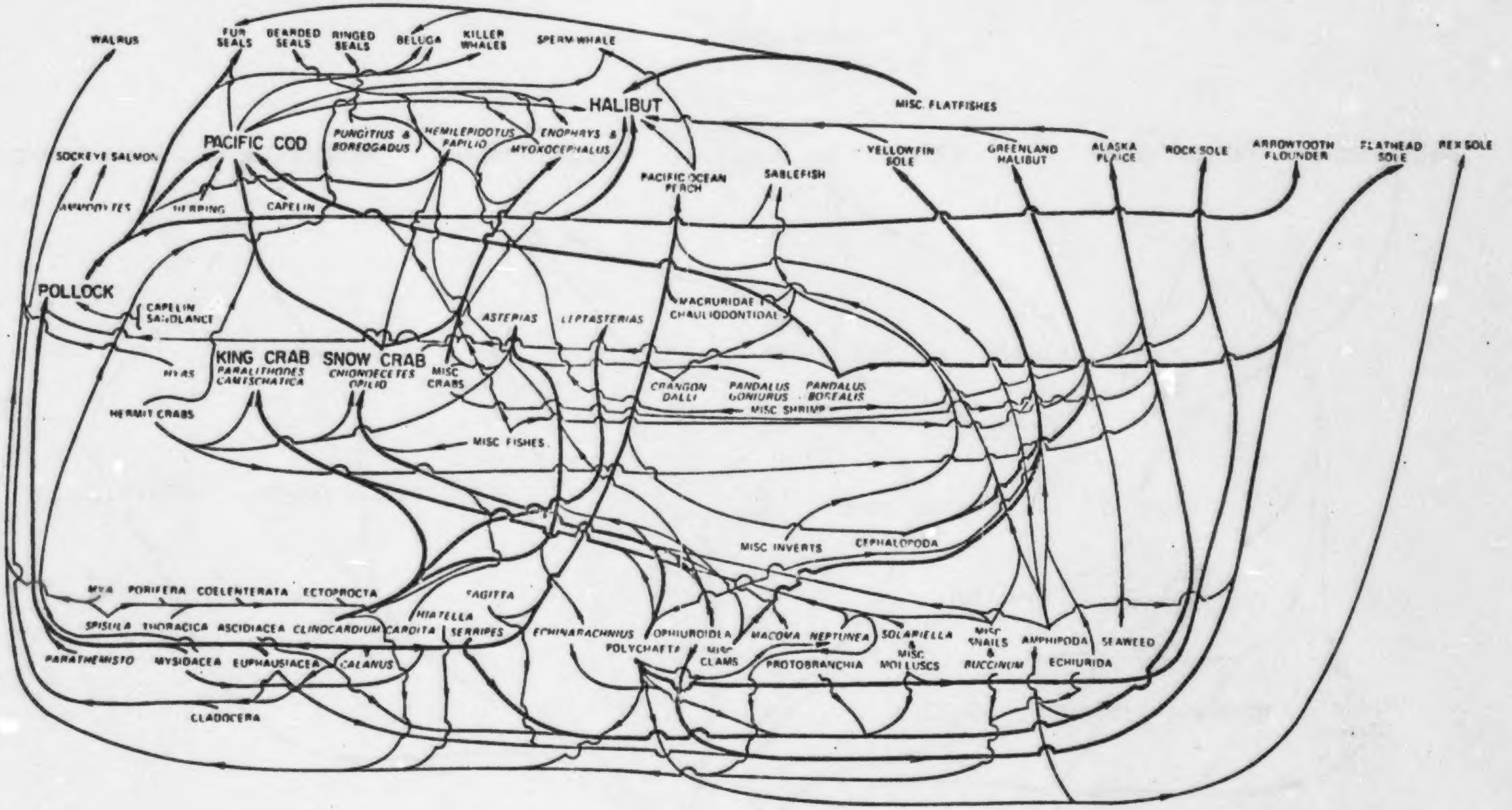

Figure 1a. A food web based on the benthic invertebrates of the southeastern Bering Sea. Carbon flows generally in the direction of the arrows. Bold lines indicate major food sources based on frequency of occurrence. (From Feder, H., Annual Report, OCSEAP RU 281.)

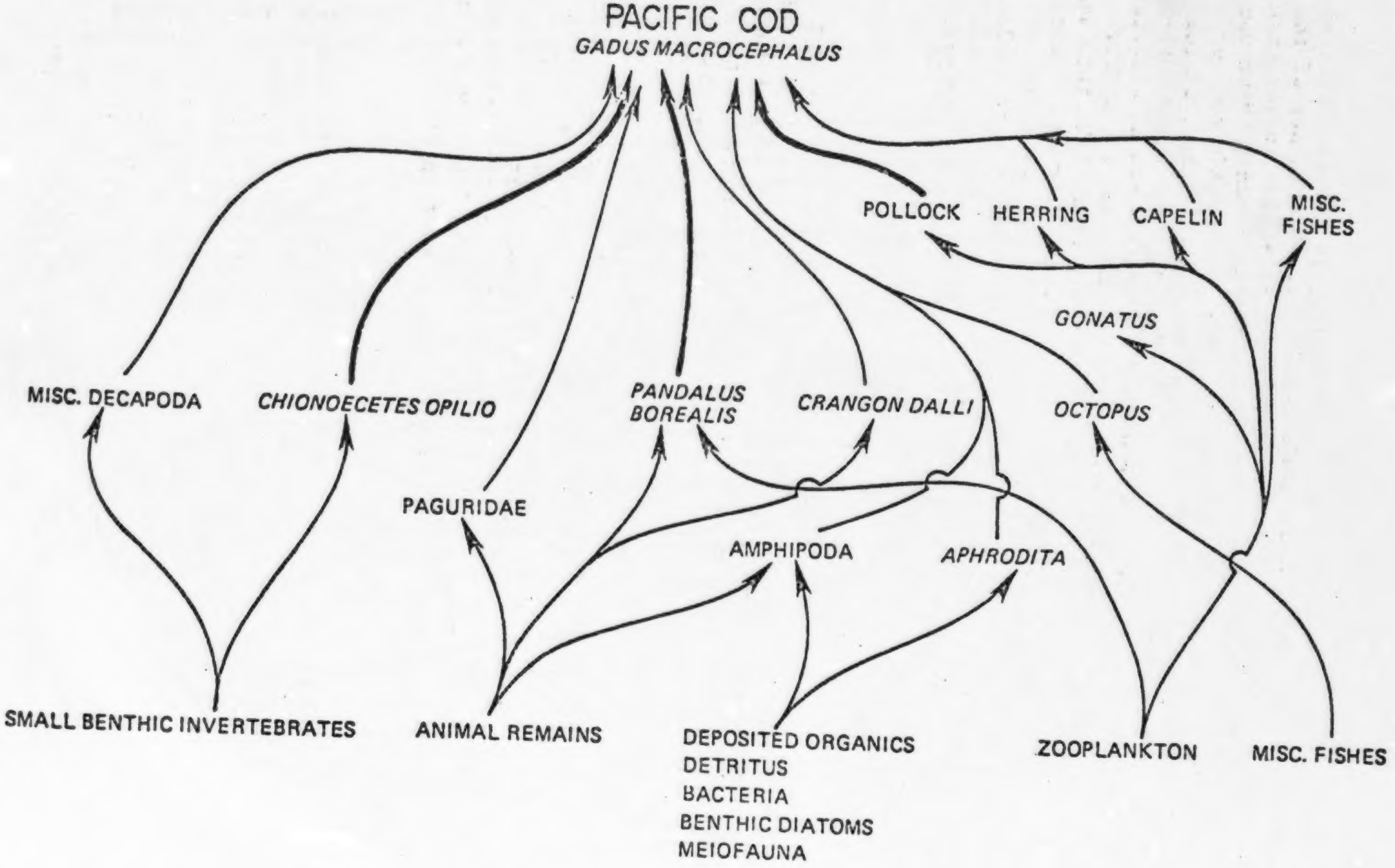

Figure 1b. A food web showing carbon flow to Pacific cod (Gadus Macrocephalus) in the southeastern Bering Sea. Bold lines indicate major food sources based on frequency of occurrence. (From Feder, H., 1977 Annual Report, OCSEAP RU 281.)

Ron Atlas:

OCSEAP has been investing in baseline studies, ie. in that sort of figure generation in a way, towards understanding the components of the ecosystem; also investing money in the laboratory toxicity studies that allow you to determine some of the potential effects. I don't think you can shut off those studies overnight and say "Hey we're ready now for the next big step!" I think though with that background coming in you can turn to the next step of observing a response to a spill and determining whether or not the effects you see are the same sorts of effects that you see in the laboratory and what order of magnitude the differences are. We can examine how the data fit into a picture like that and then go back to controlled situations and model systems and see whether you can now reproduce the observed effects and determine how other variables mediate that response. I think at some point you need to look at a community response and then come back and see whether your model, your laboratory work and your ecosystem understanding support and predict that sort of response.

Bori Olla:

I'd like to agree very much with that statement and I would just like to point out that I read in yesterday morning's newspaper that 500 gallons were spilled in Port Valdez Tuesday morning and I suggest that perhaps we should be using some crash-type approaches to develop the necessary understanding of how oil behaves and affects communities.

Wolfe:

Of course, OCSEAP has just such an effort, although it has so far been restricted to physical studies of oil spills. We have a Spilled Oil Research (SOR) Team with crews ready and equipped to respond to accidental spills on a contingency basis. Let's see, I don't know whether anybody who was at the Regional Meeting in Anchorage responded to that spill or not. It turned out that our SOR Team leader was at the Anchorage meeting at the time, and I think we made no response.

Bori Olla:

Much of what has been said is sort of déjà vu though not in terms of oil. Ten years ago we were concerned about temperature in the same kinds of theoretical discussions looking at community interactions and that sort of thing. But where it usually ends up though is that we all have a constituency or a patron system; you had a patron and your patron had a constituency. And that's a problem and hence many of us are forced into specifying species or "target organisms" that people easily recognize. For instance I think something like sea otters has a high public visibilty and the constituency is interested in that. (The constituency of course eventually gets back to Congressional appropriations and funding legislation.) It's hard to make the constituency understand that it's important not only to study the target organisms, but for example, something as simplistic as what the target organism depends on to make a living-to get that across is a difficult problem. So now if we take it many steps further and start with community interactions, it is even more difficult. And it seems to me that the budgeting is going down year after year and what we are talking about are energetic moves towards something that is going to be much more expensive. I think that no matter what's really the ultimate, we are going to get back to the idea that we are going to have to focus on the target organisms, ie., the commercial species, see if we can convince the legislators that some kinds of interaction beyond that, or either above or below, depending upon where they are in the food web, must also be investigated.

Wolfe:

OCSEAP per se may never get to that ultimate step. What I hope we will do though is continue to think about it with enough energy that we do lay a reasonable basis for getting there and that we do address some of the more important steps. How do we direct and focus our funds on the short term into those components that we deem most important? How can we do it other than inferring relationships in the natural system and deducing which links appear to be most important to the functioning of those high visibility components of the system? Do we utilize that approach all the way down into the individual species of zooplankton that Byron referred to earlier? How many different species and different trophic levels does it take to form a representative understanding in terms of effects?

Byron Morris:

There seems to be something on the order of twenty-two species of zooplankton involved. Communities are affected through effects on the individuals. But if there is an effect on the individual, it doesn't necessarily affect the community; species can be removed without major effects on the community. So it seems that even when we talk about species interactions, we're still interested ultimately in individual components, ie, whether the species is important to people. I don't see how to avoid focusing on the important species or on the prey of or predators upon those species.

Lois Killewich:

It seems to me that if trophic interactions are important, then we ought to go out and get some data on them to guide our effects studies. What do we know about these relationships?

Wolfe:

Last night at the restaurant, we passed around and discussed a compendium of results of major food items that have been observed in first, marine mammals (Table 1), secondly, pelagic and nearshore fish (Table 2), and thirdly, the benthic systems of the Bering Sea and Gulf of Alaska (Figures 1 and 2). The data were from work that is coming out of ADF and in the case of the marine mammals, from the University of Alaska in the case of the benthos. Figure 1 is Howard Feder's inferred trophic system for the benthos of the Southeastern Bering Sea. In the Southeastern Bering Sea, it would appear that the benthic community supports things like Pacific cod and halibut and pollock near the top of food web. Those are in turn preyed upon by marine mammals. In the benthic community *per se* it looks like king crab and tanner crab and Pandalus borealis, are key species. Some of the bivalve mollusks and the copepods and euphausia look like they are highly involved in supporting that system. Some of those species we have put a fair amount of effort into doing both short-term and long-term toxicity tests on. Others have received practically no attention. It seems like we ought to promote a better interaction between the investigators out in the field trying to deduce these kinds of relationships and those of you who are working in the laboratory on individual species effects, and we ought to begin to work on a few of these inferred important species so that we can have a better probability of projecting what the overall community effects will be.

Lois Killewich:

That doesn't mean we should overlook species that may be sensitive. We should try to determine which species are sensitive and then solicit work on those species. Are we going to look at the biochemistry or physiology of marine mammals and other potentially sensitive animals like Arctic species, or are we just going to do it on fish?

Stan Rice:

Everything is a trade-off, you know, why have we done the species that we have done, for example; we have done them primarily because we could find them in Auke Bay. They are at least generally representative of the animals from the Gulf of Alaska but not representative of those up in the Beaufort. If you are going to pay us to collect animals from up in the Beaufort and test them, you are suddenly going to be paying for about half travel and per diem and fewer research notes. And if you pay for a little bit of biochemistry on what's happening to oil hydrocarbons in a fur seal from the Pribiloffs, you're going to be cutting out research on up to 10 more valuable species. Those are the tradeoffs.

Table 1. Summary of major food items of marine mammals in the Beaufort Sea - *** indicates items of major importance; ** indicates items of moderate importance; * indicates items of slight importance. (From Burns, et al., OCSEAP Research Unit 232, 1977 Annual Report.)

Food Item	Bowhead Whale	Ringed Seal	Spotted Seal	Belukha Whale	Bearded Seal	Walrus
Euphausiids	***	***				
Mysids	**	**				
Hyperiid Amphipods	**	**				
Gammarid Amphipods	**	**				
Shrimps		**	**	**	*	
Crabs					***	
Isopods		**			***	*
Bivalve Molluscs					**	
Gastropod Molluscs					**	**
Cephalopods			*	**	*	*
Other Benthos					*	*
Polar Cod		***	**	**	*	***
Capelin		**	**	**	*	
Other Pelagic & Anadromous Fishes		*	***	***	*	
Demersal Fishes		*	**	*	***	

Table 2. Major food sources for pelagic and nearshore fish in three bays of Kodiak Island. (Compiled from Harris and Hartt final report, RU 485.)

First number gives the % occurrence of the food source in sampled stomachs of various fish; second number gives the % by weight of the food source.

Pink Salmon - juveniles (late May - late July is intertidal)		
Harpacticoids	70%	97%
Pink Salmon - juvenile Pink Salmon (late June - mid-September in epipelagic)		
Calanoids	61%	10%
Capelin	13%	77%
Juvenile Chum Salmon - (late May and late July in Agak and Alitak Bays)		
Harpacticoids	50%	12%
Gammarids	30%	18%
Dolly Varden		
Gammarids	23%	36%
Sand Lance (A. heraplirus)	14%	43%
Capelin		
Euphausids	25%	8%
Calanoids	21%	20%
Masked Greenling (juvenile)		
Gammarids	58%	28%
Harpacticoids	33%	9%
Polychaetes	33%	40%
Adult Masked Greenling		
Gammarids	40%	30%
Polychaetes	25%	13%
Juvenile White-Spotted Greenling from Alitak Bay		
Calanoids	71%	63%
Juvenile White-Spotted Greenling from intertidal of Alitak Bay		
Harpacticoids	66%	14%
Gammarids	43%	28%
Great Sculpin		
Gammarids	22%	1%
Snake Pricklebacks		
Gammarids	56%	60%
Harpacticoids	29%	9%

Table 2.--Continued.

Pacific Sandfish		
Misc. Retantia Zooea	75%	28%
Teleost larvae	25%	39%
Sand Lance		
Calanoids	35%	75%
Rock Sole		
Gammarids	50%	1%
Polychaetes	44%	10%
Pelecypoda	34%	8%
Yellowfin Sole		
Pelecypoda	31%	4%
Juvenile Coho Salmon		
Gammarids	73%	85%
Juvenile Pacific Cod		
Gammarids	60%	35%
Mysidacea	39%	10%
Harpacticoids	22%	1%
Starry Flounder		
Gammarids	40%	87%
Sand Sole		
Mysids	74%	94%

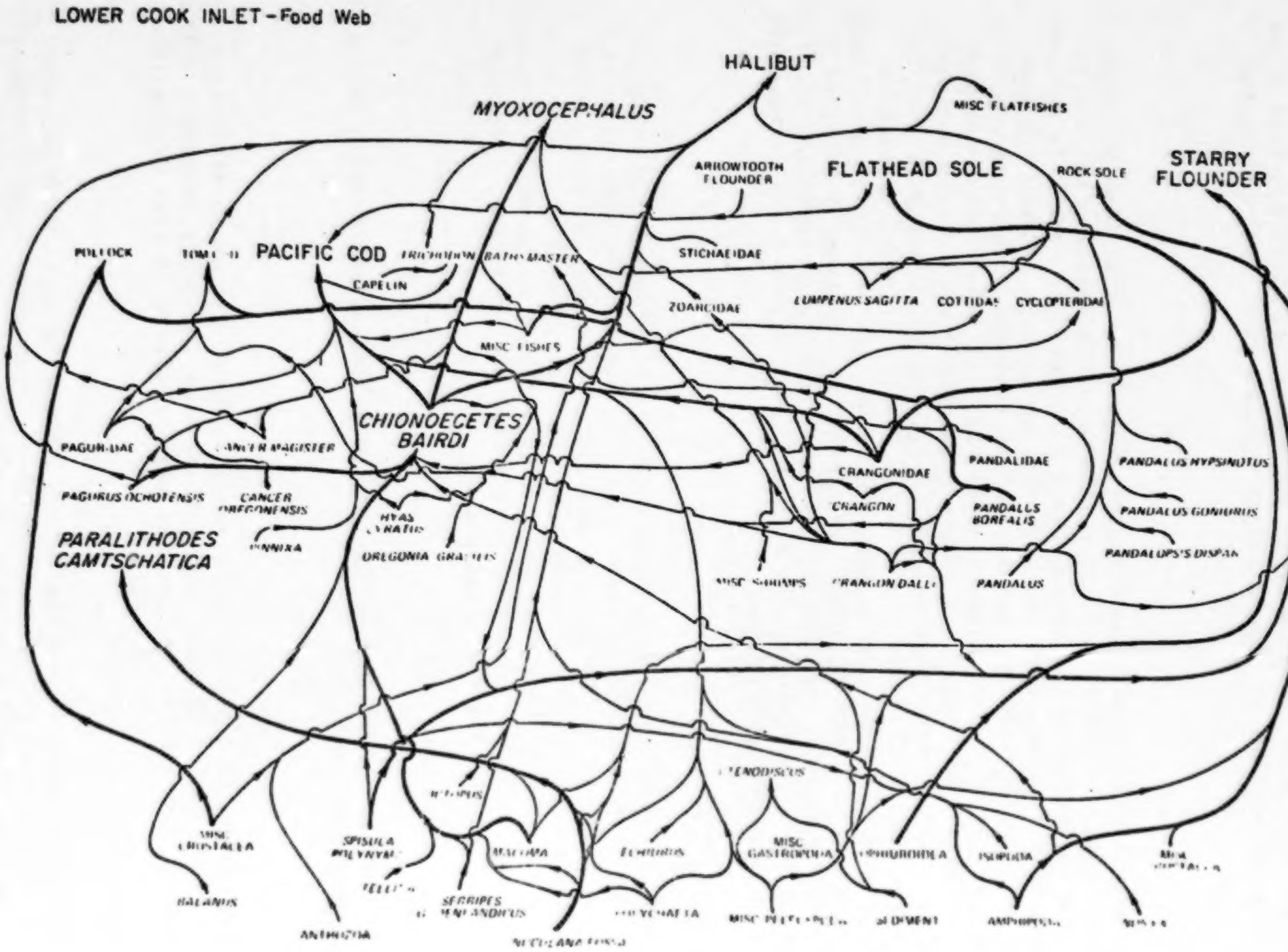

Figure 2. A food web based on the benthic invertebrates of lower Cook Inlet. Carbon flow is in the direction of the arrows. Bold lines indicate major food sources based on frequency of occurrence. (From Feder, H., 1977 Annual Report, OCSEAP RU 281.)

Lois Killewich:

That goes back to the argument you made before which is whether we're going to study species which are sensitive, which may cost a lot of money, or whether we should select species with short generation time.

Wolfe:

Again those are the two different objectives that Stan referred to before. One, we want to make sure that we are addressing the species that are important to the system based upon our best available estimate of what that importance is and secondly, we want to address species which are readily cultured and apparently taxonomically representative, so that we can draw generalizations on the kinds of effects, particularly sublethal effects, that do occur and that require very extensive study in order to derive an understanding. So there may be a distinction here between studying the relative sensitivities of species in which we want to cover a wide spectrum of species prioritizing on those that appear to be more important or sensitive, and those studies on the other hand wherein we are addressing longer term more extensive studies on chronic effects where we may want to select a very few species which are ready laboratory organisms.

Bruce McCain:

Another aspect too, is what species are most vulnerable to an oil spill? Some species remain close to the surface, other species associate with sediments on beaches and shorelines that may be contaminated and that's an important consideration. In that respect flat fish have an advantage in that they have pelagic life stages which would be floating near the surface. Also they reside near the shoreline, they bioaccumulate, and they are sensitive too.

Jack Bend:

Do you see a need in your program to differentiate between acute and chronic toxicity?

Wolfe:

Not except in a purely operational sense as far as feasibility and cost of experimental design. And I think that the criteria that I tried to mention a few minutes ago are the major criteria in that distinction.

Jack Bend:

The other point is and the reason I bring it up is with the very very low levels that you are talking about, even after a spill, compared to what we know about hydrocarbon toxicity, i.e., acute hydrocarbon toxicity in mammalian species, you are going to talk primarily about incidences of effects that are often terribly low. It seems to me that you are already placing major emphasis on those processes that are likely to be most sensitive. And these are going to be as far as I am concerned the most likely systems in which you will be able to show effect. I think if you are going to talk about studying the synergisms and the antagonisms then the only way obviously you are ever going to be able to do this is with fantastically sensitive procedures and test systems. That's why I think the behavioral things that Bori talked about and also the reproductive and sensitive life cycles are important areas of study. There is obviously still an awful lot of extrapolation between the laboratory situation and the field.

Wolfe:

And I think that large extrapolation will always be required. I'd really like to get into a discussion of that. If we can shift our thinking maybe just a little bit from the question of what species may be important. I'd like to introduce the responses to another question on the Questionnaire which I have put up here on the blackboard and those are the responses to Question number 12, which was "What aspects of the OCSEAP Effects Program should receive increased or decreased emphasis next year? in the future?" There are mind you only 12, I guess 14 now, Questionnaires that anybody responded to and it turns out that a summary of that question shows a very flat distribution, as we had 12 people responding and in effect had 12 different recommendations for both questions. In fact out of 12 respondents there was a range of zero to four recommendations for increased emphasis in the future with a median of one, and on the recommendations for decreased emphasis, again 12 responses, a range in the number of recommendations from zero to two with a median of one. So the statistics on this survey are extremely poor. But I wanted to put a few of the points on the blackboard anyway. In terms of increased emphasis in the future, there were about 5 people who in one set of words or another indicated that the effects and fate of metabolites ought to receive considerably more attention in the future. That came back on the Questionnaire in several different forms, one with the word metabolite and two with the words weathered products. The recommendation was that we should be looking at oxidation products of weathering or photooxidation or the like in terms of accumulation and turnover and effects on metabolism. Another respondent said that we should be looking at components of natural petroleum other than the things we have been looking at to date, which are benzene, napthalene, and traditional water soluble fractions. That was the single concept that seemed to have the greatest consensus. And then I coupled a number of other responses too under the concept of field experiments. Some 6 people in one form or another mentioned the desire for and the need of field experiments as an important future direction. Spills seemed to play heavily in that concept, ie, that we should be doing controlled field experiments or making experimental observations under spill circumstances. People mentioned accidental spills, to focus on under ice spills, mentioned population recovery (that's something that Ron referred to a little while ago) as an aspect of community or population transitions that we should be looking at. Somebody else indicated we should be looking at effects on food webs. I wasn't exactly sure what that meant, but it seemed to me like it probably was something that should be approached in field experiments, so if whoever said that meant something different, speak now. Those two concepts were the only two that I really thought could be lumped and then there were a number of single responses that went through a series of things like impacts on behavior, increased effects on vertebrates, increased effort on microbes. Someone else indicated that we ought to be looking at biological adaptations to chronic petroleum exposure. Considering the fact that a lot of these responses are one-person/one-recommendation sort of thing you can probably look around the room and deduce who might have responded that way. On the decreased emphasis, there were two points that recurred on three responses and those were, we should probably be looking less at acute toxicity of single organisms and we should be looking less at the effects of metals in both organisms and systems. Metals effects work was mentioned on at least two other questionnaires in terms of OCSEAP relevance as opposed to in terms of scientific interest perhaps. Finally, consistent with what we just indicated we should be looking at compounds other than benzene, napthalene and water soluble fractions. Earlier I indicated a recommendation that we should spend less time looking at the kinds of volatile low molecular weight aromatic compounds that have so dominated the bioassay work over the past six years. Then there were a series of single responses and I only chose to put one of those up on the board. One respondent recommended that we should put considerably less effort into monitoring changes in communities and populations because of the extremely low feasibility of ascribing any kind of causal relationships between those changes and any kind of environmental perturbation. I found that an interesting response in the light of the high consensus on need for increased emphasis on field research which seems to me to imply at least that the population and community changes are exactly the sort of thing that we ought to be looking at.

Jack Anderson:

Aren't you confusing monitoring with experiments?

Wolfe:

I put the word on the board in order to make sure that I didn't forget what it was that was said, but I guess somewhere in those semantics I do get confused about the difference between monitoring and measuring change. Monitoring indicates to me that you are making measurements in order to detect change, and that's sort of what I always hoped to be able to do when I design an experiment too.

Jack Anderson:

But it's the intensity of the sampling effort that allows you to do it in a limited area of study and one cannot do that in the coastal or offshore Alaska region. It's virtually statistically impossible to get enough data on distribution of organisms over that second region to use as a guide as to whether there was an impact.

Wolfe:

O.K. So what you are saying is that the concept of using population parameters as a baseline against which one detects future change that might be caused by a specific stressant, like petroleum, is not a good concept because you can't get sufficiently intensive sampling effort out there to correlate conclusively with the impact of interest. I'd like to work on that concept-I happen to agree with it.

Comment:

Perhaps the distinction is one of active versus passive activity: monitoring where you are not controlling experimentally what you are noting and in the cause of the experimental situation you have controlled active manipulation under defined circumstances what you are trying to measure, and that would define the difference between monitoring and the field experimental stages of what you are trying to do.

Ron Atlas:

Let me take another tack, and indicate that in part the original concept behind hypoxia which is a product of change was to somehow have a baseline and know what some of the effects would be and that you measure the effects against existing community structure. What Jack is saying here is that you can't do that because you are never going to establish an accurate baseline because of the spatial and seasonal sampling intensity required to do that. What we ought to do then is turn around and intensively study an area where you can achieve the necessary sampling. In which case you are going to know something about that area when you get into an actual spill and it somewhat helps. You won't have the ability then to respond in any sense, ie, in the original sense of monitoring, if you have not done any background or reconnaissance work in that area. You won't know what to begin to do.

Jack Anderson:

Well, I would answer to that that neither is going to help you. In other words, your previous observations on a spill area will probably never be intensive enough either. What one needs to do instead is to try experiments which will produce a different type of data which might allow you to predict to a degree what the impact might be. It is a difficult problem, I realize that, I think that reconnaissance has been worthwhile up to this point to get a feel for what is there, but it is not going to increase our knowledge that much compared to experimental approaches.

Wolfe:

We may conclude though on the basis of some of the preliminary reconnaissance that there are in fact selected population parameters in certain types of samples that are compatible with the monitoring concept. It seems obvious that the more uniform in time and space that a parameter is, the more compatible it is.

Ron Atlas:

The only other thing to add to that is the question of whether you have achieved reconnaissance to the point where you're satisfied you at least have some idea of what's in each of the areas we are talking about, or whether there have been gaps left in the reconnaissance where areas have never been sampled.

Wolfe:

I would still like to pursue the point because I would like to work from the concept of broad population community monitoring on a large geographic and spatial scale, back to the feasibility of looking at population community parameters, on any scale. I would like to see whether we can address the types of design criteria or limitations that we might encounter in design of a field impact experiment. If we encounter statistical problems in monitoring populations on a broad scale, where do we, as we narrow the scale, cease to have problems? What kinds of systems can we in fact, hope to look at successfully?

Bruce McCain:

I can't answer all of that but I think one of the main advantages of baseline type work is to prevent oversimplification of the effects of the oil spill. I think that is one of the main advantages. For instance in terms of our disease work, people can't oversimplify and say that I found a diseased fish, therefore this oil spill caused it. And I think that can be extrapolated to other types of baseline measurements.

Jack Anderson:

A statistician should be involved, should sit down and look at all the data available for a particular hypothesis, and then you would be able to say, is this a reasonable experiment, ie., a good design? Is this a reasonable number of individuals that we can expect to see the desired effect and conclude anything on the causality of an oil spill?

Dennis McLaughlin:

I can try to respond to that. Obviously you are going to find a lot of variability in a population, regardless of the philosophy of the experiment. The variability is what results in sometimes impractical sampling environments, impractical numbers of samples, or numbers of replicates. The real issue in limiting the experiment-looking at a smaller site or a smaller system, is to be able to explain the variability. The real problem in replication is the residual variability left over after you have tried to explain through environmental factors, for example variations in elevation and exposure and the abundance of associated species, let's say. You look at it as much as you can in the field to explain the variability, and the more you can explain, the less you have to do in the way of replication. I think one of the problems with the survey samples, or monitoring as we've been calling it here, is that you go out and just look at what's there. You notice a patch here or a patch there and you notice stratification and you just accept it and do not try to correlate it with other variables. That's one of the problems of looking at historical data. The variability of historical data may not be indicative of the variability obtained in an experiment where all identified causes of variability are measured also. Perhaps in the historical situation some rather simple thing such as beach exposure was not noted, yet that was a major reason for variability in the intertidal community.

Dick Grau:

This is a very abstract discussion, and it's hard for someone who is not really directly involved in the Alaska program to relate to it. But one possibility that might help me at least respond to it would be to say, If a situation arose right now and there were a team available to investigate a spill of say 5,000 gallons or something like that in some particular spot, what kinds of information would people want to collect from that? And what would they do with it if they got it? In other words, that's really a test of where we are right now--how much do we know, how much are we willing to extrapolate, how much value can we place on a particular type of information about selected organisms? With a lot of birds, they wouldn't even be there at certain times of the year and you wouldn't need to worry about it at all, whereas other organisms might be present all the time, therefore, cause for concern. So in some ways, to come back to reality, we might test this by setting up a modeling experiment; not a modeling study in the sense of a computer model, but a sort of scenario--what would happen if.

Wolfe:

I see this conversation has held remarkably well to the Agenda, which was to lead us through future needs and research direction into the question of how to validate laboratory observations under field circumstances. OCSEAP has already put some effort into this general problem in the form of the SOR Team, the Spilled Oil Research Team, and in the form of a contract to Resource Management Associates represented here by Dennis McLaughlin who has looked at some of the problems of design criteria and feasibility, based upon available data, for conducting controlled spills. Both of those turn out to lead very well into the topic of oil spill response and impact assessment on a national level. Some of us will be continuing to meet this afternoon to develop a set of objectives that should be pursued by the Regional Response Team for Alaska. Thank you for your participation in this discussion.

20. Reviewers' Comments and Recommendations for Future Program Direction

Six outside reviewers, each very familiar with certain aspects of the fate and effects of contaminants in marine organisms and ecosystems, were invited to attend the program review and to provide detailed comments on the quality and directions of the program. These reviewers are listed below:

Dr. Jack Bend
Nat'l Institute of Environmental
Health Sciences
Research Triangle Park
North Carolina 27711

Dr. Thomas M. Church
College of Marine Studies
University of Delaware
Newark DE 19711

Dr. Paul Falls
Atlantic-Richfield Company
P. O. Box 360
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Dr. Harry Ohlendorf
Asst Director of Patuxent
U.S. Fish and Wildlife Service
Patuxent Wildlife Research Ctr
Laurel MD 20810

Dr. David S. Peters
NOAA National Marine Fisheries

Southeast Fisheries Center
Beaufort Laboratory
Beaufort NC 28516

Dr. John J. Stegeman
Woods Hole Oceanographic Institute
Woods Hole MA 02543

The extensive detailed comments of the reviewers are not reproduced here, but have been taken into consideration, and will be applied in the "fine tuning" of individual research efforts under OCSEAP support. The following recommendations were distilled from these comments and from discussion with the reviewers, and represent major points on which there was close agreement among the reviewers on the overall content or direction of the program.

1. Tasks addressed by the effects program

The subtasks set forth in task F (see Introduction for itemization) are for the most part reasonable, and are being met completely or partially. These include F-1, F-2, F-3, F-4 and F-7. F-5, F-6 and F-8 are either not being addressed, as yet, or are not being addressed in an adequate manner. F-4 should be down-played based on results to date, and the food chain or ecosystems part of F-5 should be deleted, as it is not feasible to conduct on a small scale and because generalization from work elsewhere should be applicable to Alaska as well.

2. Need for field studies

The major weakness in the effects program is the overemphasis of laboratory studies at the expense of natural systems and studies of "real world" situations. The biochemical, cellular, tissue and organismal studies are appropriate, but not to the total exclusion of population and community research. Although the results obtained from studies of processes at the community or ecosystem levels will likely be less well defined than those at the cell or organism level, the emergent properties of populations and communities may be of direct and more immediate importance to the environmental decision-makers.

The study of marine population and community ecology is a difficult and relatively new field with little background to lead the uninitiated in designing a successful program, but the works cited by Moore and McLaughlin are evidence that such studies can be productive. Research in population and community ecology need not be characterized by high and unmanageable variation if the investigators have an appreciation of appropriate sampling techniques and also choose the population or system most appropriate for studying the properties of particular interest.

In order to answer many of the questions being addressed in OCSEAP, a combined field study would be advisable. This could take the form of a controlled spill in a region in Alaska where most of the investigators would have the opportunity to apply their techniques and interpretation in a realistic situation beyond the confines of the laboratory. Too many of the effects units are now carrying on strictly confined laboratory studies as isolated units. A real field study would provide an integrating mechanism besides showing relevancy to BLM of the research techniques being developed. The employment of criteria such as those described by Moore and McLaughline would be necessary.

A related approach is to plan an integrated field study around an accidental oil spill in Alaska, on a contingency basis. The likelihood of an accidental spill is sufficient with the opening of the Alaska pipeline to warrant formulation of a contingency study team representing most of the effects research groups. The study objectives and design would be similar to those used in a controlled spill experiment.

3. Need for analytical intercalibration

The coordination of analytical results within the effects program requires a carefully designed intercalibration of sampling and analysis. This should be not only for gaining certification by an individual laboratory but it provides an excellent means for teaching the means of carefully executed trace petroleum analysis and unifying the best methodology.

The decision to set up the national analytical facility of NOAA should guarantee consistent analytical data quality on which depends much of the interpretation of biological effects studies. This facility should be the focus for quality control within the effects program and expand its expertise beyond just hydrocarbons. The facility could also take the lead in a careful NOAA review of procedures on data and sample handling.

4. Relevance of metals work to OCS studies

Results of Anderson and Miller et al. suggest that oil impaction does not significiantly mobilize heavy metals from sediments. However, it may be fruitful to test mixtures which are either oxygenated or anoxic because the redox conditions will be critical for the extent and cause of mobilization. Also noteworthy is the lack of metal uptake from petrogenic heavy metals, which has received little attention before this study (Anderson). These results indicate that metals contaminants is not a major concern of OCS development and further work on effects of metals should be discontinued. Any work conducted on the toxicity of metals should carefully consider the speciation of naturally occurring metals and should use realistic dose levels to ensure 1) that realistic estimates of uptake and turnover rates are obtained, and 2) that the observed effects are likely to occur under environmental conditions.